## AD NUMBER

**ADB282296**

## NEW LIMITATION CHANGE

TO

**Approved for public release, distribution unlimited**

FROM

**Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Aug 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.**

## AUTHORITY

**USAMRMC ltr, dtd 15 May 2003**

AD_____

Award Number: DAMD17-99-1-9139

TITLE: Isolation of Genes Involved in Rac Induced Invasion and Metastasis of Breast Carcinoma Cells

PRINCIPAL INVESTIGATOR: Linda Van Aelst, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

## NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

## LIMITED RIGHTS LEGEND

Award Number: DAMD17-99-1-9139
Organization: Cold Spring Harbor Laboratory



THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Carol B Christian _____

6/4/02 _____

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 2001 | Annual (01 Jul 00 - 01 Jul 01) |

**4. TITLE AND SUBTITLE**
Isolation of Genes Involved in Rac Induced Invasion and Metastasis of Breast Carcinoma Cells

**5. FUNDING NUMBERS**
DAMD17-99-1-9139

**6. AUTHOR(S)**
Linda Van Aelst, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-Mail: vanaelst@cshl.org

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Distribution authorized to U.S. Government agencies only (proprietary information, Aug 01).
Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

A well-defined explanation of the progression of breast cancer to the metastatic state is still lacking at the molecular level. The involvement of Rac1, a member of the Rho GTPases, in cellular processes implicated in tumor progression, such as proliferation, adhesion and invasion, is manifest. To identify target genes of Rac1 which mediate its effects on invasion and metastasis, we applied cDNA-RDA and microarray analyses. This work resulted in the identification of 85 independent gene fragments (among them 23 novel genes) which showed altered expression levels as a result of Rac1V12 and Rac1N17 expression. The difference in mRNA abundance of twenty genes has been reconfirmed by northern blot analysis. Among the twenty genes are previously identified genes associated with tumorigenesis and/or invasion. We focussed our efforts on the characterization of cyclin D1 and COX-2 (both genes' expression levels were upregulated as a result of Rac1V12 expression) with respect to mediating Rac1's effects on breast tumor progression, and found a role for COX-2 in Rac1V12-triggered increase in cell growth. In addition, we initiated experiments to obtain full-length cDNA's of the novel isolated gene fragments, which may result in the identification of novel components involved in conferring tumorigenic phenotypic changes.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer, Rac invasion/metastasis | 108 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Van Aelst R

PI - Signature Date

Table of Contents

## INTRODUCTION

Our major goal for this proposal is to obtain a better understanding of the molecular process by which a primary tumor progresses to its metastatic state. This process is complex and consists of multiple steps. These steps include increased local proteolysis, degradation of extracellular matrix components, invasion, adhesion to and migration through the vascular basement membrane, and proliferation at distant sites (Kleiner and Stetler-Stevenson, 1999; Liotta et al., 1991; Mignatti et al., 1986; Moscatelli and Rifkin, 1988; Stetler-Stevenson et al., 1992; Stetler-Stevenson and Yu, 2001; Welch et al., 2000). Tumor progression is believed to be the result of multiple alterations, including genetic alterations and changes in gene expression, resulting in loss of normal cellular regulation (Nicolson, 1991; Nicolson, 1998). Over the past few years, members of the Rho GTPases, in particular Rac1, have emerged as key players in adhesion, invasiveness, proliferation and metastasis (reviewed in Boettner and Van Aelst, 2001; Schmitz et al., 2000) (see attachments). Hence, the identification and characterization of the target molecules mediating Rac1-specific effects on proliferation, invasion and metastasis is likely to provide more insights into the mechanism of tumor progression, as well as possibly identify additional diagnostic markers and new targets for therapy. As was outlined in last year's report, we performed cDNA-RDA (representational difference analysis of cDNA) in combination with microarray technology to identify genes which are up- or downregulated as a result of constitutively active and dominant negative Rac1 mutant expression in the breast epithelial cell line, Mcf7. We have identified 85 independent gene fragments (of which 23 were novel) that showed altered expression levels. The gene products of the known genes could be subdivided into different classes, including mitochondrial enzymes, ribosomal proteins, cell cycle components, and cytoskeleton associated proteins. Over the past year, we have been focussing our efforts on the further characterization of some of these genes with respect to their involvement in mediating Rac1's effects on breast tumor progression.

## BODY

To identify target genes of Rac1 which mediate its effects on invasion and metastasis, we proposed the following specific aims: 1) Identify genes that are differentially expressed in epithelial cells which do and do not express Rac1V12, 2) Analyze and initially characterize the isolated candidate genes, and 3) Determine a role for the candidate gene products in invasion and metastasis. As described in detail in last year's report, we had applied cDNA-RDA in combination with microarray technology, which led to the identification of several interesting potential Rac1 target genes. We have now finalized sequencing all the differentially expressed gene fragments and reconfirmed the altered expression of 20 genes by northern blot analysis. A summary is given below. Following the identification of potential Rac1 target genes, we decided to focus on two genes which particularly attracted our attention, given their previously reported involvement in tumorigenesis. These genes are COX-2 and cyclin D1. We have begun their further characterization with respect to mediating Rac1's effects on breast tumor progression. A detailed description is given below.

**Summary: Identify and initially characterize genes that are differentially expressed in breast epithelial cells which do and do not express Rac1V12.**

To identify specific genes whose levels of expression are regulated by Rac1, we had performed cDNA-RDA (representational difference analysis of cDNAs). In this technique, one cDNA population (called the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester (Hubank and Schatz, 1994; Lisitsyn and Wigler, 1993). We performed two sets of cDNA-RDA. In the first experiment, we set out to identify genes that are upregulated by Rac1V12 and Rac1N17 in breast epithelium Mcf7 cells by using cDNAs isolated from cells induced to express Rac1V12 and Rac1N17 as the tester population, and cDNAs isolated from uninduced cells as the driver population. In the second experiment, we used cDNAs isolated from Mcf7 cells induced for Rac1V12 and Rac1N17 expression as the driver, and cDNAs from uninduced cells as the tester, to identify genes which are downregulated by Rac1V12 and Rac1N17 respectively. The Mcf7 inducible cell lines expressing Rac1V12 and Rac1N17 were generated using the retroviral Tet-off system (Clontech). The Tet-off system (containing the tetracycline-controlled activator, tTA receptor) is designed such that medium containing doxycycline activates the tetracycline receptor which secondarily represses the Tet-dependent promotor. Conversely, depletion of doxycycline from the medium causes a conformational change in the tetracycline receptor, resulting in its inactivation and derepression of the target promotor. A description of retroviral transduction can be found in our recent chapter in Methods in Enzymology (Boettner et al., 2001), and a detailed protocol to perform cDNA-RDA can be found in our chapter in Methods in Molecular Biology (Schmitz et al., 2001) (see attachments).

To confirm the differential expression of the isolated difference products (DPs) obtained from the cDNA-RDA experiments, we made use of micro-array technology (in collaboration with R. Lucito at CSHL). PCR amplified inserts from the isolated DPs were printed onto a glass slide, and then driver and tester cDNAs were differentially labeled with the nucleotide derivatized fluorophores Cy3 and Cy5 during preparation of first strand synthesis with reverse transcriptase. The labeled cDNAs were hybridized to the microarray chip and the fluorescence signals resulting from the sequence specific hybridization were quantitated separately for each channel and compared to yield a ratio (Lucito et al., 2000; Schena et al., 1995). This, in other words, generated a measure of the abundance of one message as compared to the other. The clones found to be differentially expressed based on microarraying (fluorescence ratio greater than 2) were subjected to sequencing. A more detailed description can also be found in our chapter in Methods in Molecular Biology (Schmitz et al., 2001).

We obtained 85 independent gene fragments, of which 23 were novel, and a total of 37 apparently upregulated and 48 downregulated candidate genes as a result of Rac1V12 or Rac1N17 expression. The obtained genes could be categorized into the following groups: genes encoding nuclear, ribosomal, mitochondrial, membrane-associated, secreted and cytoskeletal proteins. Such a broad spectrum of genes encoding proteins of diverse classes

has also been previously observed for genome wide transcriptome screens, such as that for Ras target genes (Zuber et al., 2000).

To assure that the differences we observed, when comparing two representations of cDNAs, reflect true differences in mRNA abundance, we performed northern blot analysis. Thus far, we have examined the expression levels of 20 clones in Mcf7 cell lines expressing RacV12, RacN17, or empty vector, using [$\alpha^{32}$P] dCTP-random labeled cDNAs (from the 20 candidate genes) as probes, and reconfirmed their difference in mRNA abundance. The identities of the 20 clones and their ratios obtained from the microarray experiments are shown in Table 1.

| Rac1V12 upregulated genes | (ratio) | Rac1V12 downregulated genes | (ratio) | Rac1N17 upregulated genes | (ratio) | Rac1N17 downregulated genes | (ratio) |
|---|---|---|---|---|---|---|---|
| myosin II | 2.8 | CDO | (3.7) | protein with homology to HE-4 | (2.3) | ICAPI | (2.4) |
| calcyclin | 4.8 | VH16 | (2.2) | vesicle sorting protein VPS35 | (2.8) | | |
| hBMP-4 | (2.2) | β-1-4 galactosyltransferase | (4.8) | | | | |
| STAT5 | (2.9) | Mitochondrial cytochrome c oxidase | | | | | |
| ribosomal protein (PO) | (3.2) | (cox III) | (3.1) | | | | |
| dynein | (2.6) | EST3 | (4.2) | | | | |
| hephaestin | (7.2) | | | | | | |
| cyclin D | (3.6) | | | | | | |
| Cyclooxygenase-2 (COX-2) | (4.8) | | | | | | |
| ICAPI | (2.7) | | | | | | |
| NF-KB | (6.2) | | | | | | |
| EST1 | (3.7) | | | | | | |
| EST2 | (6.1) | | | | | | |

**Table1: Differential expression of genes in Mcf7 cells, resulting of Rac1V12 or Rac1N17 expression.** The isolated DPs obtained from the cDNA-RDA experiments were printed onto a glass slide (chip). Tester and driver cDNAs were differentially labeled and hybridized to the microarray chip. The fluorescence signals resulting from the hybridization were quantitated separately for each channel and compared to yield a ratio.

We are presently finalizing northern blot analysis for all the other clones, including the ESTs. Once this is accomplished (which we anticipate will be done within approximately one month), the information on the identity of all the clones will be made available in a manuscript we are presently preparing (Schmitz et al. in preparation). In the mean time, we have obtained all of the ESTs which matched our obtained difference products which did not appear to encode for known proteins. The further identification of their gene products may provide more insight into the molecular mechanism of tumor progression, and may reveal additional diagnostic markers. In addition, we have focussed our efforts on the characterization of COX-2 and cyclin D1 (see below).

**Determine a role for the candidate gene products in proliferation, invasion and metastasis.**

As mentioned above, two clones, namely COX-2 and cyclin D1, particularly attracted our attention as potentially being important in mediating Rac1's effects on breast tumor progression, given their previous implications in tumorigenesis. A brief summery of previous findings on COX-2 and cyclin D1 pertinent to this study is given below.

As shown in Table 1, we found that both COX-2 and cyclin D1 expression levels were upregulated as a result of Rac1V12 induction. The differences in mRNA abundance of COX-2 and cyclin D1 in Rac1V12 induced Mcf7 cells were reconfirmed by northern blot analysis and are illustrated here as examples. We extracted RNA from cell lines transiently transfected with Rac1V12 or empty vector to confirm that the altered expression of COX-2 and cyclin D1 were not a consequence of generating the Rac1V12 inducible stable cell line. As shown in Fig. 1, we found that COX-2 and cyclin D1 indeed are upregulated in Rac1V12 transfected cells compared to vector transfected cells. Similar results were obtained for the protein levels of COX-2 and cyclin D1 by western blot analysis, using extracts of the above cell lines and mouse monoclonal antibodies against COX-2 and cyclin D1 (See Fig. 2).

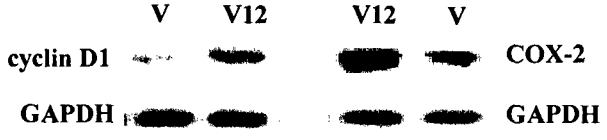



**Fig. 1: Northern blot analysis of cyclin D1 and COX-2 expression.** 15 $\mu$g of total RNA were isolated from cells transiently transfected with Rac1V12 or empty vector and assessed by northern blot analysis using cDNA probes for cyclin D1 and COX-2. Load normalization was verified by reprobing blots with a GAPDH cDNA.

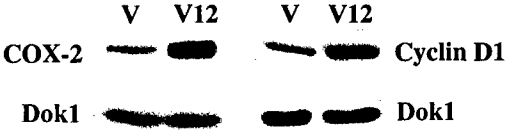



**Fig. 2: Western blot analysis of cyclin D1 and COX-2 expression.** Rac1V12- or empty vector-expressing Mcf7 cells were lysed and equal protein amounts were subjected to western blot analysis using a monoclonal Ab against COX-2 (Transduction Laboratories) or a monoclonal Ab against cyclin D1 (Pharmingen). Load normalization was verified using a polyclonal Ab against dok1

Cyclooxygenase-2 (COX-2) is the inducible isoform of prostaglandin H synthase and has been implicated in a broad range of physiological and pathological processes, including inflammation, maintenance of gastrointestinal integrity, and progression of a variety of cancers (Dubois et al., 1998; Taketo, 1998). In contrast to the isoform COX-1, which is constitutively expressed in nearly all normal tissues, COX-2 expression can be

upregulated by various stimuli, including inflammatory signals, cytokines, mitogens, and growth factors (Dubois et al., 1998; Williams and DuBois, 1996). The increase in the rate of COX-2 gene transcription is mediated by several promoter elements that respond to multiple signal transduction pathways. Increased levels of COX-2 have been demonstrated in pancreatic, gastric, esophagal, lung, head and neck, GBMs, and metastatic breast tumors (Castelli et al., 1989; Deininger and Schluesener, 1999; Higashi et al., 2000; Howe et al., 2001; Uefuji et al., 2000; Zimmermann et al., 1999). At present, the most compelling evidence for a role of COX-2 in tumorigenesis comes from the findings that several transgenic and carcinogen-induced colon tumor animals express high levels of COX-2 in intestinal polyps. Furthermore, a lack of COX-2 expression results in decreased neoplastic growth and the number of tumors that develop in APCΔ176 mutant mice (Oshima et al., 1996). The precise mechanism by which COX-2 contributes to tumorigenesis remains to be determined. However, several lines of evidence suggest that COX-2 promotes cell survival (Chang et al., 2000; Sheng et al., 1998).

Cyclin D1 is a key player in cell cycle regulation in mammalian cells. Its activity is essential for passage through the checkpoint (G1→S) of the cell cycle. Cyclin D1 abundance varies within the cell cycle, peaking in G1, and is largely controlled by the rate of cyclin D1 transcription (reviewed in Pestell et al., 1999). The latter is regulated by multiple transcription factors, including NFκB, AP-1 and STATs. The abundance of cyclin D1 is rate limiting in G1 progression, at least in part because of its role in the formation of holoenzyme complexes with CDK4 and CDK6, which phosphorylate and inactivate the retinoblastoma tumor suppressor (pRB). This causes release of pRB from the E2F transcription factor, activation of E2F responsive genes, and subsequent entry into S phase (Lukas et al., 1995; Pestell et al., 1999). The finding that cyclin D1 is upregulated in Mcf7-Rac1V12-expressing cells is consistent with our previous observation that Rac1V12 can trigger cyclin D1 induction in fibroblasts (Westwick et al., 1997).

Based on the above information and our observation that both COX-2 and cyclin D1 expression levels are upregulated by Rac1V12 in the Mcf7 cell line, we postulate that COX-2 and cyclin D1 play an important role in mediating some of Rac1's effects on Mcf7 cell growth and invasion. Before addressing this hypothesis, we measured the growth rates and invasive potential of the Mcf7 cells induced for Rac1V12 expression. As shown in Fig. 3, the growth rate of Rac1V12 induced Mcf7 cells was markedly faster than the one of uninduced control cells. Cells containing Rac1V12 were plated in 6-well-dishes at a density of $2.5 \times 10^4$ per well in medium containing 0.5 % serum with and without 25 $\mu$M doxycycline (DOX). Cells were then fixed in 10% formalin at indicated time points throughout one week, and stained with crystal violet. Cell-associated dye was extracted with acidic acid, and the optical density was measured at 590 nm, with each value normalized to day 0 (Lin et al., 1998).

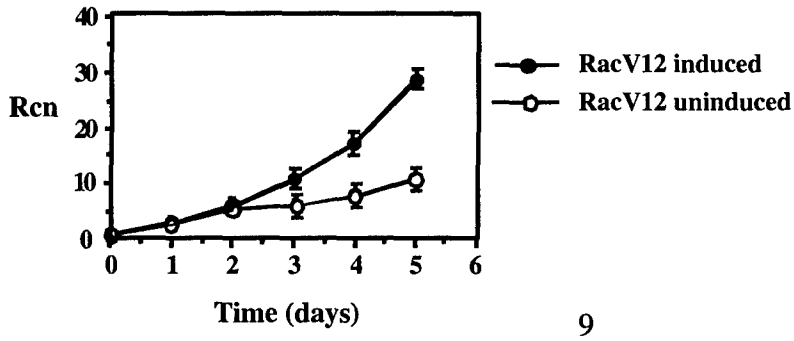



**Fig. 3: Growth properties of Rac1V12-expressing Mcf7 cells.** Growth rates of Mcf7 cells induced and uninduced for Rac1V12 expression, determined in the presence of 0.5 % serum. Each value was determined in triplicate and normalized to the cell number at day 0. (Rcn= relative cell number)

As shown in Fig. 4, induction of Rac1V12 expression also increased the invasive potential of these cells on collagen. We made use of a modified Boyden chamber assay to measure the invasive potential of cells (Banyard et al., 2000). Briefly, this assay utilized Transwell chambers (Costar), 6.5 mm in diameter with 8$\mu$m pore size polycarbonate filters. The membranes were coated with collagen overnight and then placed into 24 well tissue culture plates containing DMEM + 5% serum and 1% BSA. Uninduced cells and cells induced for Rac1V12 expression by withdrawal of DC were serum starved, harvested, resuspended in serum free medium containing 1 % BSA, and added to each Transwell chamber. The cells were then incubated for 16 hours to allow them to invade toward the underside of the membrane. Non-invading cells on the upper surface of the filter were removed with a cotton swab, while cells that had migrated to the underside of the membrane were fixed, stained with Hema 3 (Fisher), and the number of cells per membrane counted under a light microscope.

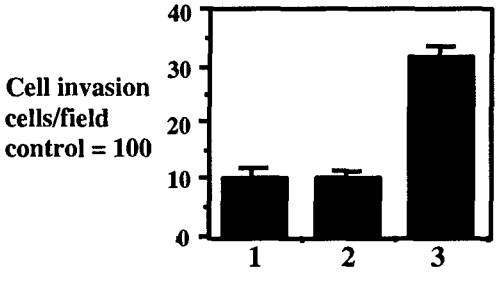



**Fig. 4: Cell invasion assay for Rac1V12-expressing Mcf7 cells.** Cell invasion through collagen was analyzed using a modified Boyden chamber assay (see text). Cells that migrated to the lower side of the membrane were counted from at least 48 different fields (63x magnification) and for three experiments. The averaged values were normalized to those obtained for the parental Mcf7 control cells (1= control; 2= Rac1V12 uninduced; 3= Rac1V12 induced.

To investigate the involvement of COX-2 in Rac1-mediated effects on cell growth, we made use of a widely used inhibitor of COX-2, NS-398 (Barnett et al., 1994; Castano et al., 2000). Mcf7-Rac1V12 cells maintained with and without DOX were treated with different concentrations (1, 5, 10, 25 and 50 $\mu$M) of the NS-398 drug, or were left untreated, and growth rates were measured as described above. We noted that the addition of 12.5 $\mu$M NS-398 could suppress the increased growth rate triggered by Rac1V12 induction, but only affected the growth rate of Rac1V12 uninduced cells slightly (Fig. 5). These data suggest that COX-2 may play a role in mediating the effects of Rac1 on Mcf7 cell proliferation. We are currently testing the effects of the drug NS-398 on the invasive potential of Rac1V12 expressing cells.

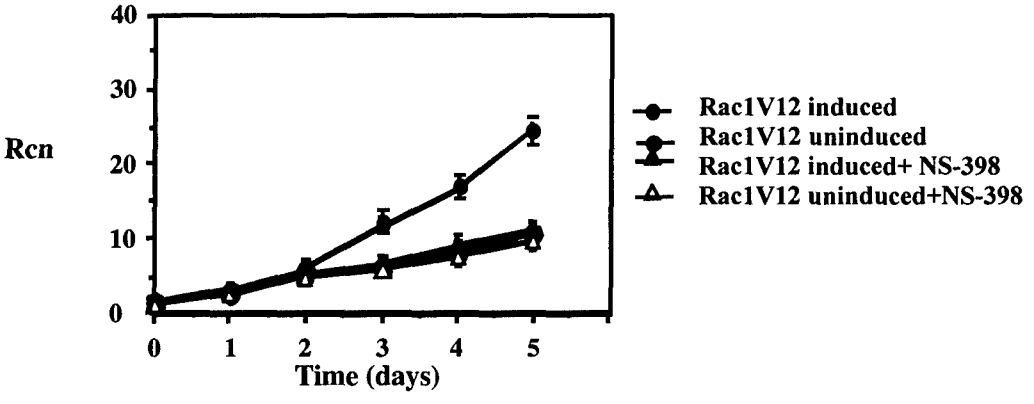



**Fig. 5: Effect of COX-2 inhibitor on the growth rate of Rac1V12-expressing Mcf7 cells.** Growth rates of Mcf7 cells induced and uninduced for Rac1V12 were treated with 25$\mu$M NS-389 or left untreated. Each value was determined in triplicate and normalized to the cell number at day 0. (Rcn = relative cell number)

To test whether interfering with cyclin D1 expression can reduce the Rac1V12-triggered increase in cell growth, we will make use of a dominant negative cyclin D1 mutant (T156A). The cyclin D1 (T156A) mutant prevents nuclear import of CDK4 and its phosphorylation by CDK-activating kinases. When expressed in cells, it competes with endogenous cyclin D1 and hence mobilizes CDK4 into cytoplasmic, catalytically inactive complexes (Diehl and Sherr, 1997). We have already constructed a retroviral dominant negative cyclin D1 (T156A) mutant construct and transduced Mcf7-Rac1V12 cells with this construct, as well as with an empty control vector. We are currently selecting for stable Rac1V12 lines expressing the dominant negative cyclin D1 (T156A) mutant and empty vector. The resulting cell lines will be assessed for altered cellular growth and invasive properties following induction of Rac1V12 expression

## KEY RESEARCH ACCOMPLISHMENTS

- We finalized sequencing all the clones found to be differentially expressed based on microarraying (fluorescence ratio greater than 2). This resulted in the identification of 85 independent gene fragments (of which 23 were novel), with altered levels of expression as a result of Rac1V12 or Rac1N17 expression.

- We completed northern blot analyses for twenty of the differentially expressed genes (see Table 1) and were able to reconfirm their altered expression levels. Among these genes, several have been previously associated with tumorigenesis. We obtained all of the ESTs for which we had noted matches with our obtained difference products and are in the process of obtaining full-length genes.

- We demonstrated that the inducible Rac1V12 Mcf7 cell line we generated shows an increased growth rate and an increased invasive potential upon induction of Rac1V12 expression. We further initiated experiments addressing the role of COX-2 and cyclin D1 in mediating Rac1's effects on breast tumor progression. We provided evidence supporting a role for COX-2 in Rac1V12-triggered increase in growth of the breast cell line, Mcf7.

## REPORTABLE OUTCOME

### Manuscripts:

Schmitz, A., Boettner, B., Govek, E.E., and Van Aelst, L. (2000). Rho-GTPases: Signaling, migration, and invasion. Special issue on cell adhesion. Exp Cell Res, 261:1-12.

Schmitz, A., Lucito, R., and Van Aelst, L. (2001). Identification of Rac-regulated genes using cDNA-RDA in combination with microarraying. Methods in Molecular Biology (in press).

Schmitz, A., Lucito, R., and Van Aelst, L. (2001). Identification of novel Rac target genes in epithelial cells (manuscript in preparation).

Boettner, B., Herrmann, C., and Van Aelst, L. (2001). Ras and Rap interaction with the AF-6 effector target. Methods in Enzymology: Regulators and effectors of small GTPases. (edited by E.E. Balch, J. Der and A. Hall) 332:151-168.

Boettner, B. and Van Aelst, L. (2001). The role of Rho-GTPase in disease development. Gene. (In press).

**Presentations (Linda Van Aelst)**

Invited speaker Seminar, McGill University, Montreal, Canada (April. 6, 2001). Host: N. Lamarche.

Invited speaker Seminar, University of Virginia, Charlottesville, USA (April 24, 2001). Host: J. Casanova.

Invited speaker FASEB Research Conference on the Ras Superfamily of Small GTP-binding proteins, Snowmass, Colorado, USA (July 15-20, 2000).

Invited speaker and chair: Tyrosine phosphorylation and cell signaling meeting, Cold Spring Harbor, New York, USA (May 16-20, 2001).

Invited speaker Oncogene meeting: Cancer cell signal transduction. Hood College, Frederick, Maryland, USA (June 20-23, 2001)

Chairperson: American Association for Cancer research. Convention Center, New Orleans, LA, USA (March 24-28, 2001)

## CONCLUSIONS

To identify target genes of Rac1, we applied cDNA-RDA and microarray analyses, which resulted in the identification of 85 independent gene fragments (among them 23 novel genes) which showed altered expression levels as a result of Rac1V12 or Rac1N17 expression. The obtained genes could be categorized into several different groups: genes encoding nuclear, ribosomal, mitochondrial, membrane associated, cell cycle, secreted and cytoskeletal proteins. To reconfirm the differential expression of the above genes, we performed northern blot analyses. Thus far, we reconfirmed the difference in mRNA abundance of twenty genes (see Table 1), most of which were previously identified. Of particular interest is the observation that many of these genes have been previously demonstrated to play a role in tumorigenesis and/or invasion. We focussed our initial attention on the characterization of cyclin D1 and COX-2 with respect to mediating Rac1's effects on breast tumor progression, and obtained evidence supporting a role for COX-2 in Rac1V12-triggered increase in cell proliferation. Further experiments addressing the importance of the two molecules above in Rac1-triggered effects on invasion are under way.

Based on our current data, we also believe that amongst the 23 novel genes identified, several will be involved in conferring invasive and metastatic potential during different stages of the tumor progression process. Hence, we will also devote a great deal of effort to obtaining their full-length cDNAs. These genes may provide additional diagnostic markers, as well as new targets for therapy.

## REFERENCES


Banyard, J., Anand-Apte, B., Symons, M., and Zetter, B. R. (2000). Motility and invasion are differentially modulated by Rho family GTPases. Oncogene *19*, 580-591.

Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osen, E., Nguyen, B., Tsing, S., Bach, C., Freire, J., and et al. (1994). Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. Biochim Biophys Acta *1209*, 130-139.

Boettner, B., Herrmann, C., and Van Aelst, L. (2001). Ras and Rap1 interaction with AF-6 effector target. Methods Enzymol *332*, 151-168.

Boettner, B., and Van Aelst, L. (2001). The role of Rho-GTPase in disease development. Gene *in press*.

Castano, E., Bartrons, R., and Gil, J. (2000). Inhibition of cyclooxygenase-2 decreases DNA synthesis induced by platelet-derived growth factor in Swiss 3T3 fibroblasts. J Pharmacol Exp Ther *293*, 509-513.

Castelli, M. G., Chiabrando, C., Fanelli, R., Martelli, L., Butti, G., Gaetani, P., and Paoletti, P. (1989). Prostaglandin and thromboxane synthesis by human intracranial tumors. Cancer Res *49*, 1505-1508.

Chang, Y. W., Jakobi, R., McGinty, A., Foschi, M., Dunn, M. J., and Sorokin, A. (2000). Cyclooxygenase 2 promotes cell survival by stimulation of dynein light chain expression and inhibition of neuronal nitric oxide synthase activity. Mol Cell Biol *20*, 8571-8579.

Deininger, M. H., and Schluesener, H. J. (1999). Cyclooxygenases-1 and -2 are differentially localized to microglia and endothelium in rat EAE and glioma. J Neuroimmunol *95*, 202-208.

Diehl, J. A., and Sherr, C. J. (1997). A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin- dependent kinase 4 (CDK4) and its phosphorylation by CDK-activating kinase. Mol Cell Biol *17*, 7362-7374.

Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. Faseb J *12*, 1063-1073.

Higashi, Y., Kanekura, T., and Kanzaki, T. (2000). Enhanced expression of cyclooxygenase (COX)-2 in human skin epidermal cancer cells: evidence for growth suppression by inhibiting COX-2 expression. Int J Cancer *86*, 667-671.

Howe, L. R., Subbaramaiah, K., Brown, A. M., and Dannenberg, A. J. (2001). Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. Endocr Relat Cancer *8*, 97-114.

Hubank, M., and Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucl Acids Res *22*, 5640-5648.

Kleiner, D. E., and Stetler-Stevenson, W. G. (1999). Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol *43*, S42-51.

Lin, A. W., Barradas, M., Stone, J. C., Van Aelst, L., Serrano, M., and Lowe, S. W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes & Dev *12*, 3008-3019.

Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell *64*, 327-336.

Lisitsyn, N., and Wigler, M. (1993). Cloning the differences between two complex genomes. Science *259*, 946-951.

Lucito, R., West, J., Reiner, A., Alexander, j., Esposito, D., Mishra, B., Powers, S., Norton, L., and Wigler, M. (2000). Detecting gene copy number fluctuations in tumor cells by microarray analysis of genomic representations. Genome Res.

Lukas, J., Bartkova, J., Rohde, M., Strauss, M., and Bartek, J. (1995). Cyclin D1 is dispensable for G1 control in retinoblastoma gene- deficient cells independently of cdk4 activity. Mol Cell Biol *15*, 2600-2611.

Mignatti, P., Robbins, E., and Rifkin, D. B. (1986). Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell *47*, 487-498.

Moscatelli, D., and Rifkin, D. B. (1988). Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. Biochim Biophys Acta *948*, 67-85.

Nicolson, G. L. (1991). Gene expression, cellular diversification and tumor progression to the metastatic phenotype. Bioessays *13*, 337-342.

Nicolson, G. L. (1998). Breast cancer metastasis-associated genes: role in tumour progression to the metastatic state. Biochem Soc Symp *63*, 231-243.

Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996). Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell *87*, 803-809.

Pestell, R. G., Albanese, C., Reutens, A. T., Segall, J. E., Lee, R. J., and Arnold, A. (1999). The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr Rev *20*, 501-534.

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science *270*, 467-470.

Schmitz, A., Lucito, R., and Van Aelst, L. (2001). Identification of Rac responsive genes using cDNA-RDA and micro-array technology. Meth Mol Biol *In press*.

Schmitz, A. A., Govek, E. E., Bottner, B., and Van Aelst, L. (2000). Rho GTPases: signaling, migration, and invasion. Exp Cell Res *261*, 1-12.

Sheng, H., Williams, C. S., Shao, J., Liang, P., DuBois, R. N., and Beauchamp, R. D. (1998). Induction of cyclooxygenase-2 by activated Ha-ras oncogene in Rat-1 fibroblasts and the role of mitogen-activated protein kinase pathway. J Biol Chem *273*, 22120-22127.

Stetler-Stevenson, W. G., Liotta, L. A., and Brown, P. D. (1992). Role of type IV collagenases in human breast cancer. Cancer Treat Res *61*, 21-41.

Stetler-Stevenson, W. G., and Yu, A. E. (2001). Proteases in invasion: matrix metalloproteinases. Semin Cancer Biol *11*, 143-152.

Taketo, M. M. (1998). Cyclooxygenase-2 inhibitors in tumorigenesis (part I). J Natl Cancer Inst *90*, 1529-1536.

Uefuji, K., Ichikura, T., and Mochizuki, H. (2000). Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. Clin Cancer Res *6*, 135-138.

Welch, D. R., Steeg, P. S., and Rinker-Schaeffer, C. W. (2000). Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. Breast Cancer Res *2*, 408-416.

Westwick, J. K., Lambert, Q. T., Clark, G. J., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. (1997). Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol Cell Biol *17*, 1324-1335.

Williams, C. S., and DuBois, R. N. (1996). Prostaglandin endoperoxide synthase: why two isoforms? Am J Physiol *270*, G393-400.

Zimmermann, K. C., Sarbia, M., Weber, A. A., Borchard, F., Gabbert, H. E., and Schror, K. (1999). Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res *59*, 198-204.

Zuber, J., Tchernitsa, O. I., Hinzmann, B., Schmitz, A. C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schafer, R. (2000). A genome-wide survey of RAS transformation targets. Nat Genet *24*, 144-152.

## APPENDICES

Manuscript: Schmitz, A.P., Govek, E-E., Böttner, B. and Van Aelst, L. (2000) Rho GTPases: signaling, migration, and invasion.

Schmitz, A.P., Lucito, R., and Van Aelst, L. (2001) Identification of Rac-regulated genes using cDNA - representational difference analysis (cDNA-RDA) in combination with microarraying.

Böttner, B. and Van Aelst, L. (2001) The role of Rho-GTPases in disease development.

Böttner, B., Herrmann, C., and Van Aelst, L. (2001) Ras and Rap1 interaction with AF-6 effector target.

Experimental Cell Research **261,** 1–12 (2000)
doi:10.1006/excr.2000.5049, available online at http://www.idealibrary.com on IDEAL®

# MINIREVIEW

# Rho GTPases: Signaling, Migration, and Invasion

Arndt A. P. Schmitz, Eve-Ellen Govek, Benjamin Böttner, and Linda Van Aelst[1]

*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724*

**The acquisition of a motile and invasive phenotype is an important step in the development of tumors and ultimately metastasis. This step requires the abrogation of cell–cell contacts, the remodeling of the extracellular matrix and of cell–matrix interactions, and finally the movement of the cell mediated by the actin cytoskeleton. Evidence for participation of Rho GTPases in migration and invasion is addressed in this review with emphasis on epithelial cells and the contribution of Rho GTPases toward tumor invasion. The Rho GTPases, including Rac, Cdc42, and Rho, have been implicated in the establishment of cell–cell contacts and of cell–matrix interactions crucial to attaining a fully polarized epithelial state, and they are known for their regulation of the actin cytoskeleton and transcriptional activation. Under aberrant conditions, however, they have been implicated in motility, invasion, and some aspects of metastasis. It is well known that Rho GTPases are activated by different classes of transmembrane receptors and that they transmit these signals to their effector proteins. These downstream targets include not only adaptor proteins and kinases which affect the actin cytoskeleton, but also transcription factors leading to expression of genes necessary for the drastic morphological changes which accompany these processes.** © 2000 Academic Press

*Key Words:* **Rho GTPases; signaling; motility; actin cytoskeleton; invasion.**

## INTRODUCTION

The acquisition of migratory and invasive properties are key events in the oncogenic progression of cells [1]. For example, while an untransformed adherent cell undergoes a particular form of apoptosis known as anoikis after a loss of adhesion, the activity of certain oncogenes appears to be able to overcome this effect. In addition, oncogenic disregulation, apart from a proliferative hyperactivity, can induce a switch in epithelial cells from a stable adherent phenotype to a motile and invasive one. Epithelial cells establish stable contacts between adjacent cells, and between individual cells and the extracellular matrix, thus maintaining a fully polarized state. Most malignant tumors are epithelial in origin, and transition from a normal to an invasive phenotype requires drastic reprogramming on a genetic and physiological level, known as epithelial-to-mesenchymal transition [2]. This transition results in (a) both abolition and transitory activation of adhesive abilities of the cell, (b) remodeling of the actin cytoskeleton, (c) recognition of chemotactic and haptotactic cues, and (d) proteolytic processing and secretion of extracellular matrix (ECM) proteins along the trajectory. In order to perform these changes, cells need to activate a variety of signaling pathways and to change their transcriptional profiles (Fig. 1).

In order to illuminate recent advances made toward understanding the effects Rho GTPases play in this complex scenario of adhesion, migration, and invasion, we first need to explicate the specific molecular functions affected by these processes. An epithelial cell in a normal situation is laterally linked to its akin neighbors and basally rooted in the ECM, which it either produces itself or is provided by an underlying fibroblast cell layer. Cell linkage is mediated by cell–cell adhesion complexes such as tight and adherens junctions (TJs and AJs respectively), the former junctions being established more apically in the lateral membrane than the latter. Both of these junctional entities are hallmarks of fully polarized cells and indispensable for the function and maintenance of this state. Fully polarized cells are distinguished by an apical membrane domain exposed to the lumen of a particular organ or tissue, a lateral membrane domain that is linked to other cells in the layer, and a basal domain tightly linked to the ECM by focal adhesions (FA) [3].

Cell–cell and cell–matrix signaling and adhesion processes are mediated by specific cell adhesion molecules (CAMs) [4]. The principle CAM of an epithelial cell's AJs is E-cadherin. The extracellular portion of E-cadherin engages in homophilic interactions with

[1] To whom correspondence and reprint requests should be addressed at Cold Spring Harbor Laboratory, Demerec Building, 1 Bungtown Road, P.O. Box 100, Cold Spring Harbor, NY 11724. Fax: (516) 367-8815. E-mail: vanaelst@cshl.org.



0014-4827/00 $35.00

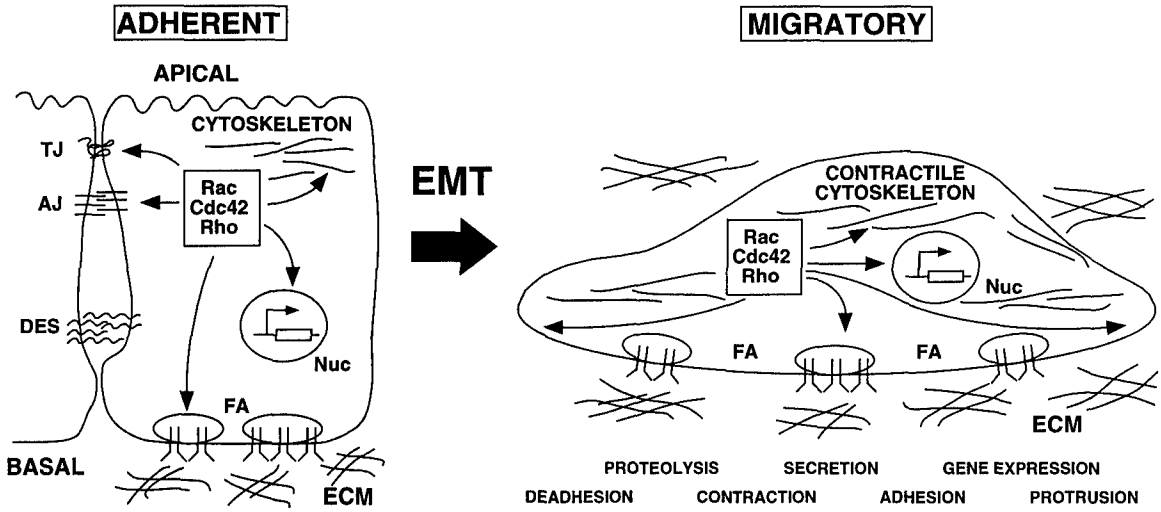



**FIG. 1.** Acquisition of a motile and invasive phenotype by an adherent epithelial cell. Rho GTPase function is required for both the establishment of a fully polarized state (left) and a motile phenotype upon epithelial-to-mesenchymal transition (EMT; right). Movement involves a number of individual steps which include the following: loss of cell–cell adhesion, membrane protrusions in the direction of movement, de-adhesion from the extracellular matrix in the rear and re-adhesion in front, contraction of the actin cytoskeleton in order to move the body of the cell, and active remodeling of the ECM by proteolysis and secretion. These steps also depend on alterations in the transcriptional profiling of the cell. (Nuc, nucleus; DES, desmosomes).

molecules of contacting neighbors, thus establishing an adherent layer which is subsequently sealed by the formation of TJs, in order to prevent paracellular flow of liquid and ions from one side of the sheet to the other. The intracellular portion of E-cadherin undergoes complex formation which links the protein to the actin cytoskeleton.

In addition to E-cadherin, a growing number of transmembrane proteins and cytoskeletal linkers have been found to play an important role in cell–cell and cell–matrix signaling. Transmembrane proteins transmit the effects of bound ligands through their cytoplasmic tails to intracellular signaling pathways, and these tails may also serve to anchor proteins to the actin cytoskeleton. The transmembrane component of focal adhesions consists of a heterodimeric integrin complex, consisting of an $\alpha$ and a $\beta$ chain, which bind to ECM proteins, such as collagen, fibronectin, vitronectin, and laminin. Ligated and activated integrin dimers assemble an elaborate adhesion and signal-promoting complex at their C-terminal juxtamembrane portion [5]. In addition, growth factor receptors of the receptor tyrosine kinase (RTK) superfamily, such as receptors for epidermal growth factor (EGF) or hepatocyte growth factor/scatter factor (HGF/SF), as well as seven-transmembrane G-protein-coupled receptors (GPCRs), such as the lysophosphatidic acid (LPA) receptor, mediate chemotactic cues [6, 7].

Rho family GTPases, including Rho, Rac, and Cdc42, have been found to mediate all of the above processes in various ways [8, 9]. Rho GTPases are required for the assembly of functional cell–cell contacts (AJs and TJs) and for their disassembly during motility. Rho GTPases control individual aspects of the actin cytoskeleton through distinct effector proteins. During the movement of the cell through the ECM, the individual steps are coordinated in a highly complicated and not yet fully understood manner. This process requires a defined level of activity and proper spatiotemporal regulation of each of the Rho GTPases, as well as cross-talk between them.

Like all GTPases, Rho GTPases act as molecular switches which cycle between an inactive GDP-bound and an active GTP-bound state, and the ratio between these two forms is dependent upon the activity of regulatory factors. GTPase-activating proteins (GAPs) promote the inactive state of a GTPase by increasing the GTPase's intrinsic rate of nucleotide hydrolysis, while guanine nucleotide dissociation inhibitors (GDIs) interfere with both the exchange of GDP for GTP and the hydrolysis of bound GTP. Guanine nucleotide exchange factors (GEFs) promote the active GTP-bound state and tether the GTPases to specific subcellular locations in order to generate an active signal. GTP-bound GTPases then associate with downstream effectors that trigger particular cellular responses [10, 11]. While dominant active mutants such as Cdc42V12, RacV12, and RhoV14 correspond to the permanently GTP-bound state, dominant negative mutants (Cdc42N17, RacN17, and RhoN19) bind more tightly to GEFs than the wild-type GTPases, but do not bind to effector proteins [12].

In this article, we summarize some intriguing recent findings that provide molecular insights into how signaling by Rho GTPases results in a motile and invasive

phenotype, focusing our attention on epithelial cells in a malignant context.

## RAC AND CDC42 SIGNALING

The Rho GTPases have been implicated in a wide variety of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity, and transcriptional activation. They are best known for their distinct effects on the actin cytoskeleton [9]. In classical Swiss 3T3 fibroblast studies, activation of Cdc42 leads to the formation of filopodia, Rac results in the formation of lamellipodia and membrane ruffling, and Rho causes the formation of stress fibers [13]. The cytoskeletal rearrangements caused by activation of Rho GTPases play a key role in the process of cell motility. It is the adhesion, subsequent loss of attachment, and re-adhesion of lamellipodia and filopodia at a cell's leading edge to the substratum which result in the coordinated and polarized movement of a cell (Fig. 1) [14–16]. On the other hand, Rac and Cdc42 also play a role in cell–cell adhesion in epithelial cells in addition to their effects on the actin cytoskeleton and motility. Expression of a dominant active form of Rac in MDCK cells or keratinocytes leads to an increase in E-cadherin complex members and filamentous actin (F-actin) at cell–cell contacts, while a dominant negative mutant was found to disrupt cell–cell adhesions [17–19]. In addition, recent studies have shown that Cdc42 plays an important role in establishing the initial polarization of epithelial cells, ultimately resulting in the formation of proper cell–cell adhesions, the disassembly of which is required for motility. For example, the introduction of a dominant negative mutant form of Cdc42 into Madine–Darby canine kidney (MDCK) cells results in the selective depolarization of basolateral membrane proteins due to inhibition of membrane transport [20]. Expression of a dominant active Cdc42 mutant in MDCK cells increased AJs and prevented cellular migration induced by HGF/SF [21]. As further discussed below, it is important to note that effects of Rac and Cdc42 in a cell are cell-type, stimulus, substratum, and concentration dependent. Given the importance of Rac and Cdc42 in the cellular events described above, perturbation of the natural balance of these GTPases in a cell may ultimately lead to phenotypes of invasion and metastasis.

The process of invasion involves a number of discrete steps leading to the attainment of this state. It involves alterations in cell–cell and cell–substrate adhesion, remodeling of the ECM, reorganization of the actin cytoskeleton, and an increase in cell motility. The first evidence of a role for Rac in invasion was obtained when the Rac-specific GEF Tiam-1 (T-lymphoma invasion and metastasis) was identified in a retroviral insertional mutagenesis screen. Virus-infected T-lymphoma cells were selected repeatedly for *in vitro* invasion through a layer of fibroblasts and the proviral insertions in invasive clones were used to identify the Tiam-1 gene [22]. Subsequently, Rac, and later Cdc42, were shown to also confer an invasive potential to these T-lymphoma cells [23, 24]. Tiam-1 becomes activated upon localization to the plasma membrane, presumably via binding of its PH domain to the lipid products of PI3-K [25, 26]. Recent experiments have shown that activation of Tiam-1 increases the motility and *in vitro* invasion of a murine breast cancer epithelial cell line, SP-1, by mediating the effects of hyaluronic acid (HA) downstream of the HA receptor, CD44, and the cytoskeletal protein ankyrin [27, 28]. However, in contrast to the above studies, Tiam-1 has been shown to inhibit migration of NIH3T3 fibroblasts through fibronectin-coated filters [29] and to abrogate HGF/SF-induced scattering in Ras-transformed MDCK cells [30]. These effects have since been shown to be ECM dependent. On fibronectin and laminin, Tiam-1/Rac signaling inhibits invasion, while on collagen it favors motility [26].

Further evidence for involvement of Rac and Cdc42 in cellular processes pertaining to motility and invasion has since been provided. For example, expression of the laminin-receptor $\alpha 6\beta 4$ integrin in the breast carcinoma cell line MDA-MB-435 promotes invasiveness in a Rac and PI3-kinase-dependent manner [31]. Also, activated mutant forms of Rac and Cdc42 in T47D breast carcinoma epithelial cells induce invasion through a collagen matrix. Surprisingly, however, this invasion can be blocked by PI3-K inhibitors, suggesting that PI3-kinase acts downstream of Rac and Cdc42 in this system [32]. More recent evidence for Rac in cell motility comes from fibroblasts deficient for the tumor suppressor gene Pten. *Pten*$^{-/-}$ cells are more motile and contain higher levels of Rac*GTP and Cdc42*GTP than wild-type cells, and the motile behavior of these cells can be suppressed by dominant negative mutant forms of Rac and Cdc42 [33]. Since Pten is a lipid phosphatase which hydrolyzes PI(3,4,5)P3 generated by PI3-K [34, 35], these experiments not only provide evidence for Rac and Cdc42 in cell motility, but also implicate PI3-kinase in this process.

Finally, numerous groups have established a role for Rho GTPases in integrin-mediated motility. Upon binding to ECM proteins, integrins assemble a focal adhesion complex, containing cytoskeletal proteins such as talin, $\alpha$-actinin, vinculin, and paxillin, as well as focal adhesion kinase (FAK). Activated FAK then recruits Src family kinases, such as Src and Fyn, to the FA, resulting in the phosphorylation of paxillin [5]. Expression of dominant negative forms of the Rho GTPases has been shown to interfere with these processes [36]. Furthermore, a complex of adaptor proteins has been shown to mediate integrin-dependent signaling

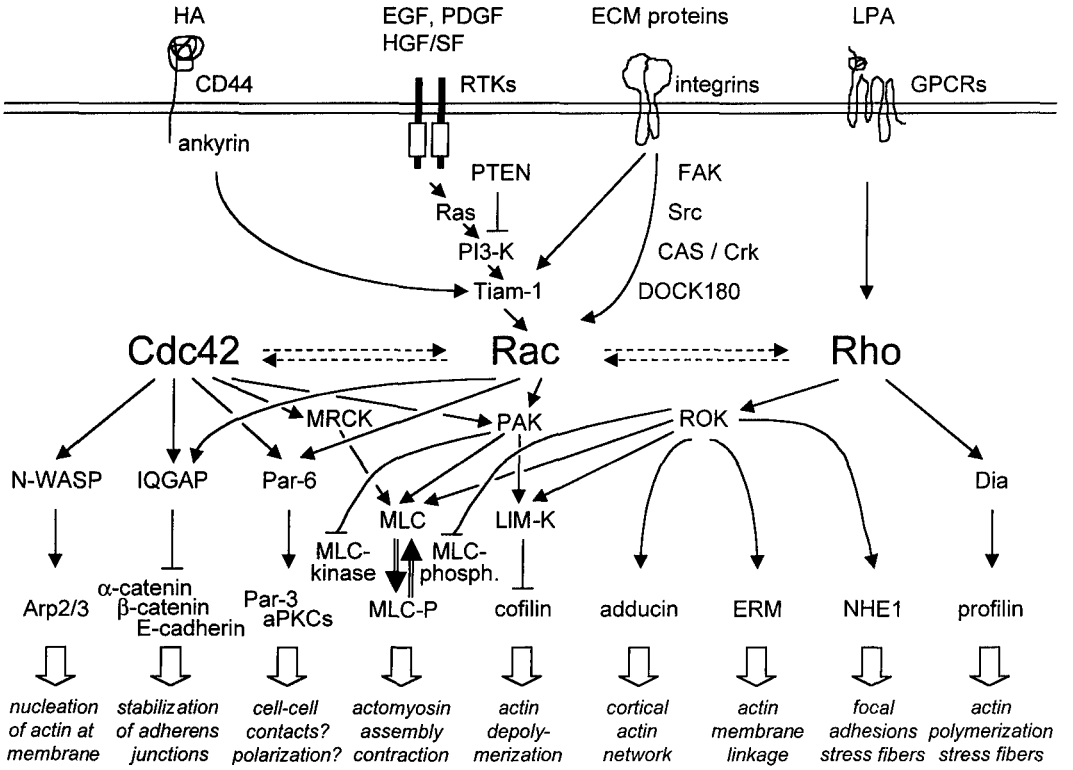



**FIG. 2.** Regulators and effectors of the Rho GTPases. Transmembrane receptors and their ligands (top) activate Rho GTPases through GEFs such as Tiam-1 or adaptor proteins. Activated Rho GTPases bind to and activate protein kinases, such as members of the MRCK, PAK, or ROK families, or to scaffolding proteins. These effector proteins then interact with several proteins with distinct effects on the actin cytoskeleton and cellular morphology (bottom). The spatiotemporal regulation of the individual interactions results in the overall control of motility. The cross-talk between the Rho GTPases is indicated by dashed arrows. Note that in particular the equilibrium between myosin light chain (MLC) and phospho-MLC (double arrows) is tightly regulated. See text for details.

upstream of Rac. FAK and Src kinases are involved *in vivo* in the phosphorylation of the adaptor protein CAS, and phosphotyrosine residues of CAS are then recognized by the SH2 domains of the Crk proto-oncogene. When expressed in COS cells, CAS and Crk cooperate in stimulating migration toward vitronectin, which is abrogated by dominant negative RacN17 [37]. In addition, Crk can bind to the docking protein DOCK180 [38] (which was shown to activate Rac [39]), and DOCK180 can enhance the migration-promoting potential of CAS and Crk [40].

## EFFECTORS OF RAC AND CDC42

Numerous effectors of Rac and Cdc42 which mediate some of the cellular activities described above have been identified (Fig. 2). Some effectors have been found to specifically mediate cell motility, whereas others play a more prominent role in cell adhesion, thus further suggesting involvement of Rho GTPases in these processes under normal and aberrant conditions. As discussed below, WASP and MRCKs serve as Cdc42-specific effectors crucial to actin organization and formation of filopodia and thus a motile phenotype. In addition, members of the p21-activated kinase family (referred to as PAK) are protein kinases downstream of Rac and Cdc42 which play an important role in cytoskeletal-mediated changes which affect motility. The scaffolding proteins IQGAP and Par-6, which can both be activated by either Cdc42 or Rac, promote cell polarization and contribute to cell–cell adhesion. Perturbation of these molecules, and thus cell–cell contacts, suggests that they may promote motility through disruption of the normal organization of neighboring cells.

As mentioned above, the ubiquitously expressed and neuronally enriched N-WASP is a scaffolding protein specific for Cdc42 [41–43]. Moreover, it binds to the Arp2/3 complex of actin-nucleating proteins crucial for the localized assembly of an actin network within filopodia [44, 45, 114, 115]. It was recently shown that both N-WASP and the Arp2/3 complex are necessary for Cdc42 to trigger actin filament assembly, and reconstitution experiments using purified proteins *in vitro* have shown that maximal actin polymerization requires not only Arp2/3, N-WASP, and Cdc42*GTP, but also the phospholipid PI(4,5)P2 [46]. Thus, through

proper filopodia formation, N-WASP may promote cellular motility.

MRCKs $\alpha$ and $\beta$ are Cdc42-specific effectors which can phosphorylate myosin light chain (MLC) via a ROK-like kinase domain [47]. Phosphorylation of MLC is required for the assembly of actomyosin complexes and their contraction, and it is the equilibrium between MLC and phospho-MLC, mainly regulated by the opposing effects of MLC-kinase (MLCK) and MLC-phosphatase (Fig. 2), which plays a pivotal role in generating force to translocate the cell body during movement. In addition, overexpression of MRCK$\alpha$ with Cdc42 synergizes to promote filopodia formation, while a MRCK$\alpha$ kinase-deficient mutant inhibits the formation of Cdc42-induced filopodia [47]. Therefore MRCKs appear to play an important role in cytoskeletal organization and contraction, which contribute to the process of migration.

PAK is a protein kinase downstream of Rac and Cdc42 [48] which plays a crucial role in actin dynamics and adhesion, and there are at least three ways through which it exerts its physiological effects. These include phosphorylation of MLCK and LIM-kinase, as well as focal complex disassembly. First, PAK has been demonstrated to phosphorylate and hence inactivate MLCK [49], resulting in a decrease in MLC phosphorylation. Thus, inactivation of MLCK results in the disassembly of stress fibers and focal adhesions, suggesting potential for motility. Indeed, decreased phosphorylation of MLC in kidney cells expressing activated PAK results in a decrease in cell spreading on fibronectin [49]. Also, invasion by highly invasive breast cancer cells, MDA-MB-435, into a gel matrix substrate is abrogated by expression of a kinase-defective PAK mutant [50]. However, PAK has also been observed to phosphorylate MLC in human microvascular endothelial cells (HMEC-1), and in this case, it is presumed that while PAK is not required for the formation of lamellipodia, it still plays a role in cell adhesion and contraction, thus contributing to cell motility [51]. In addition, an increase in MLC phosphorylation was also found in PAK-expressing NIH3T3 fibroblasts, resulting in increased directionality of haptotatic movement through a collagen gradient [52]. Secondly, PAK may control the actin cytoskeleton through the phosphorylation and subsequent activation of LIM-kinase. Phospho-LIM-kinase further phosphorylates and thereby inactivates the actin-depolymerizing protein cofilin, thus inhibiting actin depolymerization when Rac is activated and causing extreme membrane ruffling suggestive of a motile phenotype [53–55]. Thirdly, pathways involving PAK have become even more intricately interwoven with the discovery of PIX, also known as Cool [56, 57]. Manser *et al.* have provided data supportive of PIX being a Rac GEF [56]. PIX interacts with PAK and causes it to localize to focal contacts (FCs) which are integrin-dependent sites linked to the actin cytoskeleton [56]. Dissolution of FCs is necessary in order for a cell to migrate, and this is believed to occur via interaction of PIX with the G-protein-coupled receptor kinase-interacting protein (GIT1), which when overexpressed causes a loss of paxillin from FCs and promotes migration [58]. GIT1 also directly couples FAK, known to be involved in FC turnover, to FCs. Interaction of PAK with PIX leads to an increase in PAK's activity [56], and interaction of PIX with PAK allows for the formation of a trimeric GEF/GTPase/effector complex with a built-in positive feedback loop and mutual regulation of activity [116]. It also links PAK to FAK through GIT1, promoting FC turnover and thus motility [58]. Altogether, the Rac effector PAK appears to play a vital role in promoting Rac-dependent motility through a number of different regulatory mechanisms and signaling cascades.

The IQGAP1 and IQGAP2 scaffolding effectors of Cdc42 and Rac [59, 60] may regulate cell–cell adhesion through actin polymerization and sequestration of the adhesion complex molecule $\beta$-catenin. *In vitro,* IQGAP oligomerizes and cross-links F-actin [61] and it has been found to complex with Cdc42 and F-actin *in vivo* [60]. In addition, one study has shown that the IQGAP protein also competes with $\alpha$-catenin for binding to $\beta$-catenin, thus preventing attachment of the E-cadherin/$\alpha$-catenin/$\beta$-catenin complex to the actin cytoskeleton and thereby disrupting cell–cell contacts. Furthermore, the same study showed that Cdc42* GTP prevented IQGAP1 from disrupting cell–cell contacts, possibly by binding IQGAP1, and preventing it from associating with $\beta$-catenin [62]. Hence, IQGAP may provide a molecular mechanism by which Rac and Cdc42 can regulate cell–cell adhesion.

Another scaffolding protein, Par-6, is the most recently identified shared Cdc42 and Rac effector. Par-6 was identified using activated Cdc42 and TC10 mutants as baits in yeast two-hybrid screens [63, 64]. Par-6 binds to a second scaffolding protein, Par-3, and both Par-6 and Par-3 bind independently to atypical protein kinase C (aPKC) isoforms [63–65]. Most interestingly, endogenous Par-3 localizes to TJs in MDCK cells, while overexpression of Par-6 or the N-terminal portion of Par-3, the region responsible for Par-6 interaction, disrupts their formation. In addition, high expression levels of full-length Par-3 cause cell spreading beneath surrounding untransfected cells, while still maintaining a small area of tight-junctional attachment [64]. Thus we can speculate that aberrant, hyperactive Cdc42 signaling may facilitate a motile phenotype by disrupting cell–cell tight-junctional contacts. The Par-6/aPKC complex was also shown to be downstream of Rac1 and to potentiate the transforming effect of Rac1 [63]. This may be explained by a loss of cell–cell contacts as observed in the above study

[64], which could result in a loss of contact inhibition and excessive growth.

Alterations in cell morphology, such as reorganization of the actin cytoskeleton by Rac and Cdc42 above, are initiated when a cell receives a signal from its environment, resulting in a signaling cascade from receptor to nucleus, in which changes in gene expression ultimately take place. Changes in expression of proteins such as matrix metalloproteinases, including collagenase-1 (CL-1) and stromelysin-1, are vital to effecting cytoarchitectural rearrangements, since they are crucial for remodeling the ECM [66]. Kheradmand *et al.* studied the role Rho GTPases play in integrin-induced expression of CL-1 in rabbit synovial fibroblasts. They found that an increase in CL-1 resulting from disruption of $\alpha5\beta1$ integrin adhesion was dependent on Rac1, since a dominant negative form of Rac1 blocked expression of CL-1. In addition, an activated mutant form of Rac1 was sufficient to increase CL-1 expression, and this induction resulted in the generation of reactive oxygen species and was dependent on the nuclear factor $\kappa$B and interleukin-1, which acts in an autocrine manner to induce CL-1 [67]. In addition, Rac was also found to play a role in AP-1 regulation of a multigenic invasion program [68]. Interestingly, promoters of metalloproteases contain a binding site for AP-1 transcription factors, which are composed of heterodimers of the Fos and Jun protein families and are regulated by MAPK pathways [69]. In a human squamous cell carcinoma line, which generally exhibits an invasive phenotype upon EGF stimulation, it was found that a dominant negative mutant form of c-Jun is capable of preventing translocation of Rac to the membrane, thereby preventing its activation. In addition, expression of this mutant also disrupted lamellipodia formation and membrane ruffling, as well as motility and invasion [68]. From these studies, we can conclude that the physiological effects of Rac discussed above are dependent not only upon protein–protein interactions and posttranslational modifications, but also upon protein expression, and that invasion is dependent upon an intricate reprogramming of the cell.

It is evident from the studies described above that both Rac and Cdc42 play an important role in motility and adhesion, utilizing a plethora of signaling molecules. In addition, there is evidence that they play a role in invasion and thus contribute to an oncogenic state. It is noteworthy that in the process of invasion, events distinct from motility may also contribute to an invasive phenotype [70]. It appears that fine-tuned regulation of these GTPases and their interacting molecules is necessary to maintain a healthy cell state, and their effects within a cell are determined by numerous factors, such as concentration, cell type, stimulus, substrate, localization, and timing.

## RHO SIGNALING

Rho was first implicated in the formation of stress fibers and FA complexes [13], resulting in actomyosin assembly and contractile tension, two processes necessary for the forward movement of a cell. Rho has since been shown to play an important role in cell–cell adhesion. In particular, inactivation of RhoA by C3 transferase, which ADP-ribosylates the Asn41 residue, disrupts organization of actin filaments at cell–cell contacts, thus inhibiting the proper formation of both AJs and TJs [17, 18, 71, 72]. For example, in normal mammary epithelial cells, MCF10 cells, inhibition of Rho by C3 transferase disrupts E-cadherin cytoskeletal links in AJs and blocks the formation of new AJs [72]. Whereas Rho is required for the assembly of AJs, Zhong *et al.* further demonstrated that high levels of activated Rho in Ras-transformed cells contribute to the fibroblastic phenotype of these Ras-transformed epithelial cells [72]. As detailed below, the balance between contractility and cell–cell adhesion, in addition to GTPase concentration, will determine whether a cell takes on a static or a motile phenotype. Depending on different variables, such as cellular context, stimulus, and extracellular matrix, Rac and Cdc42 activation have been reported to either promote or antagonize Rho's function. In Swiss 3T3 fibroblasts, the Rho GTPases have been placed in a hierarchical order in which Cdc42 activates Rac, and Rac activates Rho ([13]; see also [8]), while in N1E-115 neuroblastoma and MDCK cells, constitutively activated Rac downregulates Rho [73, 74]. Therefore, cross-talk and a balance between individual Rho GTPases, as well as varying internal and external cellular factors, may result in a particular response [75].

There are several lines of evidence that directly link Rho to the acquisition of migratory, invasive, and metastatic phenotypes. RhoA has been implicated in an $\alpha6\beta4$ integrin-mediated pathway which is regulated by cAMP, and, somewhat surprisingly, this pathway results in the formation of lamellipodia and migration in Clone A colon carcinoma cells. Expression of a dominant negative form of RhoA resulted in the attenuation of membrane ruffling and lamellipodia formation, as well as migration, and RhoA localization to lamellipodia was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting cAMP-dependent protein kinase activity [76]. Furthermore, activation of Rho, such as by LPA, and stimulation of the actomyosin system have been associated with the transmigration of tumor cells. For example, in a metastasis assay, NIH3T3 fibroblasts expressing an activated mutant form of RhoA were injected into the tail vein of nude mice and resulted in metastasis in the lung [77]. Furthermore, in the absence of serum, activated RhoA has been found to promote invasion of cultured rat MM1

hepatoma cells through a mesothelial cell monolayer [78], and cells implanted into the peritoneal cavity resulted in invasion of the peritoneum and established tumors [78, 79]. Finally, a RhoC isoform was recently identified in a microarray-based screen for genes overexpressed in metastatic melanomas. Involvement of RhoC in metastasis was confirmed by transducing poorly metastatic A375P cells with RhoC and noting enhanced metastasis when reintroduced into mice. These cell lines were also tested for migratory and invasive potential, and it was found that introduction of RhoC into the A375P cell line resulted in an increase in migration and invasive behavior [80].

## EFFECTORS OF RHO

Members of at least two protein families appear to be required for Rho-induced assembly of stress fibers and FAs, the Rho-associated kinases (henceforth referred to collectively as ROK) and the Dia members of the formin homology family. These include the Rho effectors p164ROK$\alpha$ (Rho-associated kinase), p160ROK$\beta$ (Rho-associated coiled-coil containing protein kinase or ROCK) [81–83], Dia1, and Dia2 [84, 85]. Activation of ROK appears to be necessary, but is not sufficient, for stress fiber formation. Inhibition of ROK, using the inhibitor Y-27632, prevents stress fiber formation [86], while a constitutively activated mutant of ROK$\alpha$ merely promotes the formation of stellate actomyosin filaments, not stress fibers, in MDCK and HeLa cells [87, 88, 100]. It appears that synergy between ROK and Dia is required for proper formation of stress fibers [89, 90, 100]. Dia may contribute to stress fiber formation through its interaction with profilin, a G-actin binding protein which promotes actin polymerization and organization of actin filaments into stress fibers [84, 85, 89].

At least six substrates of ROK may also play a role in actin cytoskeletal reorganization and thus contribute to adhesion (see Fig. 2). As discussed below, these include MLC, the myosin-binding subunit of MLC phosphatase, LIM-kinase, adducin, the ERM (ezrin/radixin/moesin) family of proteins, and a $Na^+/H^+$ exchange protein (NHE1). Rho-kinase members encode for Ser/Thr kinases and have been demonstrated to phosphorylate and thus influence the activity of these molecules. The effects of three of these ROK effectors, namely MLC-phosphatase, MLC, and LIM-kinase, are best known and have been found to play an important role in driving ROK's physiological function on the actin cytoskeleton. ROK has been shown to phosphorylate, and thereby inactivate, MLC phosphatase, thus resulting in an increase in phosphorylated MLC [91, 92]. In addition, ROK can also phosphorylate MLC directly [93]. An increase in phosphorylated MLC results in enhanced actomyosin assembly and therefore an increase in stress fiber formation, adhesion, and contractility. This activity of ROK opposes inactivation of MLCK by Rac and Cdc42 via PAK (see above) and may explain apparent antagonistic effects between both Cdc42 and Rac and Rho in some cell types [94]. ROK also causes stabilization of filamentous actin through its phosphorylation of LIM-kinase, which subsequently phosphorylates and thus inactivates cofilin [95]. As mentioned above, LIM-kinase has also been shown to be phosphorylated by PAK, thus revealing yet another potential point of convergence for Rac and Rho pathways.

The ROK effectors adducin and the ERM family of proteins provide more direct regulation of the actin cytoskeleton. ROK has been shown to phosphorylate adducin [96, 97], which, together with spectrin, is an important component of the cortical actin network underlying the plasma membrane [98]. ROK-phosphorylated adducin interacts with F-actin, and its localization suggests that it may be important in the migration of a cell. In tetradecanoylphorbol 13-acetate or HGF/SF-stimulated MDCK cells, phosphoadducin localizes to membrane ruffles, and ROK-phosphorylated adducin localizes to the leading edge of migrating NRK49F fibroblasts in a wound-healing assay [97]. Furthermore, introduction of nonphosphorylatable adducin into MDCK and NRK49F cells inhibited membrane ruffling and migration in assays similar to the ones mentioned above, as did a dominant negative ROK mutant and C3 exoenzyme [97]. Finally, a constitutively active adducin mutant overcame inhibition of membrane ruffling by a dominant negative ROK mutant [97]. ROK can also phosphorylate ERM proteins important for linking actin filaments to the plasma membrane, and in this manner may affect cell adhesion and motility as well [99; however, see also 101]. Interestingly, more recently the TSC1 tumor suppressor hamartin has been demonstrated to control cell adhesion to the cell substrate through the ERM family of actin-binding proteins and RhoA [102]. Finally, a sixth ROK substrate, namely NHE1, is a ubiquitous $Na^+/H^+$ exchange protein which potentiates stress fiber formation [103].

ROK recently also has been found to increase activity of phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5-K) and subsequently increase phosphatidylinositol 4,5-bisphosphate, PI(4,5)P2, levels in HEK-293 epithelial cells [104]. PI(4)P5-K is a kinase known to generate the lipid signaling molecule PI(4,5)P2, which is involved in the Rho signal transduction pathway [105]. In particular, PI(4,5)P2 has been found to be necessary for stress fiber formation, as well as actin polymerization and perhaps FA assembly [105, 106]. Through its interaction with these different substrates named above, ROK influences the cytoskeletal structure of a cell and promotes the formation, organization,

and contraction of actin filaments in order to ultimately effect functions of Rho known to be involved in cell morphology.

Evidence exists which suggests that ROK plays a role in tumor cell invasion and metastasis. As mentioned above, activated RhoA has been found to promote invasion of cultured rat MM1 hepatoma cells through a cell monolayer [78]. In a similar assay, a dominant active form of p160ROK was also found to confer invasive potential to MM1 cells in the absence of serum and activation of Rho, while a kinase-defective ROK decreased invasive activity. In addition, the ROK-specific kinase inhibitor Y-27632 blocked Rho-induced actomyosin activation, as indicated by inhibition of MLC phosphorylation, as well as the invasive phenotype. Furthermore, continuous exposure to this inhibitor reduced the dissemination of MM1 cells implanted into the peritoneal cavity of syngeneic rats, as well as incidences of tumor nodules [79]. Further evidence implicating ROK in metastasis comes from the study of human cell lines found to play a role in intrahepatic metastasis of human hepatocellular carcinoma (HCC) [107]. When highly intrahepatic metastatic HCC cell lines were examined *in vitro,* they were found to exhibit a high degree of motility which was upregulated by LPA and inhibited by C3 exoenzyme, suggesting that activation of the Rho signaling pathway is necessary for motility in these aberrant cells. In addition, stable transfection of these cell lines with dominant negative and active forms of p160ROK also affected motility *in vitro.* Dominant negative transfectants exhibited a decrease in motility, while dominant active transfectants showed an increase in motility even in the absence of serum. Also, orthotopic implantation of intrahepatic metastatic HCC cells stably expressing a dominant negative mutant form of ROK resulted in a decrease in the metastatic ability of this cell line, suggesting that ROK is necessary for metastasis in this system [107].

As in the case of Rac, drastic changes in cellular morphology, which result from activation of Rho, most likely require extensive transcriptional reprogramming of the cell in addition to posttranslational modifications. Serum response factor (SRF) is a MADS box transcription factor which regulates cellular immediate early genes and skeletal, smooth, and cardiac muscle genes [108, 109]. It has been shown that serum and LPA-induced SRF activation is dependent upon RhoA, as is constitutively active SRF at muscle-specific promoters [110–112]. An *in vivo* screen for SRF activators identified LIM-kinase-1 as a strong SRF activator [113]. While LIM-kinase-1 is not essential for serum-induced activation of SRF, it has been shown to cause F-actin aggregation, which is dependent upon phosphorylation of cofilin, and it is regulation of actin treadmilling which actually activates SRF [113]. In addition, activated forms of mDia1 and mDia2 are both capable of activating SRF, while microinjection of an anti-mDia1 antibody into serum-deprived cells containing a stably integrated SRE-regulated reporter gene inhibited SRF activation by serum [117]. These studies therefore provide a direct link between Rho-induced actin-cytoskeletal rearrangements and gene transcription.

It is evident from the above studies that Rho and numerous downstream molecules play an important role in contractility and adhesion under normal conditions. In addition, there is growing evidence that Rho and its effector, ROK, are directly involved in invasion and metastasis. The ROK substrate adducin has been directly implicated in migration. It appears that fine-tuned regulation of Rho and related molecules is vital for a healthy cell state, and their effects within a cell are determined by numerous factors, such as concentration, cell type, stimulus, substrate, localization, and timing.

## CONCLUSIONS

The findings referred to in this article provide evidence for participation of Rho GTPases in the migration and invasion of deregulated cells. Many of the essential functions performed by Rho proteins on cytoskeletal and transcriptional levels can be oncogenically corrupted and may result in a typical tumor phenotype. The fact that a number of oncogenes encode exchange factors for the small Rho GTPases, Rho, Cdc42, and Rac, in addition to the recent observation that RhoC is transcriptionally upregulated under invasive conditions, further reinforces this notion.

Since the activating mechanisms and molecular pathways of these molecular switches are diverse in nature and context, and the effects probably even more varied, it is to be expected that there will be much more to learn. Discrepancies as to the function of a molecule often arise due to cell type, stimulus, substrate, timing, protein localization, and experimental design differences. New molecules upstream and downstream of those already implicated in tumor invasion and metastasis will undoubtedly be identified in the future, further elucidating the mechanisms by which these processes are carried out. Furthermore, identification of the spectrum of genes instrumental in the materialization of a fully invasive behavior, which are transcriptionally activated by Rho GTPases, will also shed light on cancerous states.

Because of space limitations, we are not able to cite the work of many of our colleagues who have made valuable contributions to this field. This work was supported by grants from the U.S. Army and the NIH to Linda Van Aelst, who is also a Kimmel Foundation and V Foundation award recipient. Arndt A. P. Schmitz is supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft

(DFG, Bonn, Germany). Eve-Ellen Govek was supported by a SUNY at Stony Brook institutional NIH training grant from the Department of Molecular and Cellular Biology (MCB).

## REFERENCES


1. Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100,** 57–70.
2. Birchmeier, W., and Birchmeier, C. (1995). Epithelial–mesenchymal transitions in development and tumor progression. *EXS* **74,** 1–15.
3. Rodriguez-Boulan, E., and Nelson, W. J. (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* **245,** 718–725.
4. Aplin, A. E., Howe, A., Alahari, S. K., and Juliano, R. L. (1998). Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol. Rev.* **50,** 197–263.
5. Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. *Annu. Rev. Cell. Dev. Biol.* **12,** 463–518.
6. Wells, A. (2000). Tumor invasion: Role of growth factor-induced cell motility. *Adv. Cancer Res.* **78,** 31–101.
7. Moolenaar, W. H. (1999). Bioactive lysophospholipids and their G protein-coupled receptors. *Exp. Cell Res.* **253,** 230–238.
8. Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* **11,** 2295–2322.
9. Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514.
10. Böttner, B., and Van Aelst, L. (1999). Rac and Cdc42 effectors. *Prog. Mol. Subcell. Biol.* **22,** 136–158.
11. Bishop, A. L., and Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem. J.* **348,** 241–255.
12. Feig, L. A. (1999). Tools of the trade: Use of dominant-inhibitory mutants of Ras-family GTPases. *Nat. Cell Biol.* **1,** E25–E27.
13. Nobes, C. D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81,** 53–62.
14. Lauffenburger, D. A., and Horwitz, A. F. (1996). Cell migration: A physically integrated molecular process. *Cell* **84,** 359–369.
15. Mitchison, T. J., and Cramer, L. P. (1996). Actin-based cell motility and cell locomotion. *Cell* **84,** 371–379.
16. Anand-Apte, B., Zetter, B. R., Viswanathan, A., Qiu, R. G., Chen, J., Ruggieri, R., and Symons, M. (1997). Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. *J. Biol. Chem.* **272,** 30688–30692.
17. Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H., and Takai, Y. (1997). Regulation of cell–cell adhesion by rac and rho small G proteins in MDCK cells. *J. Cell Biol.* **139,** 1047–1059.
18. Braga, V. M., Machesky, L. M., Hall, A., and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell–cell contacts. *J. Cell Biol.* **137,** 1421–1431.
19. Jou, T. S., and Nelson, W. J. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J. Cell Biol.* **142,** 85–100.
20. Kroschewski, R., Hall, A., and Mellman, I. (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. *Nat. Cell Biol.* **1,** 8–13.
21. Kodama, A., Takaishi, K., Nakano, K., Nishioka, H., and Takai, Y. (1999). Involvement of Cdc42 small G protein in cell–cell adhesion, migration and morphology of MDCK cells. *Oncogene* **18,** 3996–4006.
22. Habets, G. G. M., Scholtes, E. H. M., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP–GTP exchangers for Rho-like proteins. *Cell* **77,** 537–549.
23. Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. (1995). A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* **375,** 338–340.
24. Stam, J. C., Michiels, F., van der Kammen, R. A., Moolenaar, W. H., and Collard, J. G. (1998). Invasion of T-lymphoma cells: Cooperation between Rho family GTPases and lysophospholipid receptor signaling. *EMBO J.* **17,** 4066–4074.
25. Michiels, F., Stam, J. C., Hordijk, P. L., van der Kammen, R. A., Ruuls-Van Stalle, L., Feltkamp, C. A., and Collard, J. G. (1997). Regulated membrane localization of Tiam1, mediated by the $NH_2$-terminal pleckstrin homology domain, is required for Rac-dependent membrane ruffling and c-Jun $NH_2$-terminal kinase activation. *J. Cell Biol.* **137,** 387–398.
26. Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kammen, R. A., Michiels, F., and Collard, J. G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* **143,** 1385–1398.
27. Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2000). CD44 interaction with tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. *J. Biol. Chem.* **275,** 1829–1838.
28. Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2000). Ankyrin–Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. *J. Cell Biol.* **150,** 177–192.
29. Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999). Rac downregulates Rho activity: Reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* **147,** 1009–1022.
30. Hordijk, P. L., ten Klooster, J. P., van der Kammen, R. A., Michiels, F., Oomen, L. C., and Collard, J. G. (1997). Inhibition of invasion of epithelial cells by Tiam1–Rac signaling. *Science* **278,** 1464–1466.
31. Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997). Activation of phosphoinositide 3-OH kinase by the $\alpha 6\beta 4$ integrin promotes carcinoma invasion. *Cell* **91,** 949–960.
32. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390,** 632–636.
33. Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H., and Wu, H. (2000). Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Curr. Biol.* **10,** 401–404.
34. Myers, M. P., and Tonks, N. K. (1997). PTEN: Sometimes taking it off can be better than putting it on. *Am. J. Hum. Genet.* **61,** 1234–1238.

35. Di Cristofano, A., and Pandolfi, P. P. (2000). The multiple roles of PTEN in tumor suppression. *Cell* **100,** 387–390.

36. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998). Integrin-mediated signals regulated by members of the Rho family of GTPases. *J. Cell Biol.* **142,** 573–586.

37. Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresh, D. A. (1998). CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J. Cell Biol.* **140,** 961–972.

38. Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. (1998). Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex. *J. Biol. Chem.* **273,** 24479–24484.

39. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998). Activation of Rac-1 by a Crk SH3-binding protein, DOCK180. *Genes Dev.* **12,** 3331–3336.

40. Cheresh, D. A., Leng, J., and Klemke, R. L. (1999). Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J. Cell Biol.* **146,** 1107–1116.

41. Miki, H., Miura, K., and Takenawa, T. (1996). N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *EMBO J.* **15,** 5326–5335.

42. Aspenström, P., Lindberg, U., and Hall, A. (1996). Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott–Aldrich syndrome. *Curr. Biol.* **6,** 70–75.

43. Kolluri, R., Tolias, K. F., Carpenter, C. L., Rosen, F. S., and Kirchhausen, T. (1996). Direct interaction of the Wiskott–Aldrich syndrome protein with the GTPase Cdc42. *Proc. Natl. Acad. Sci. USA* **93,** 5615–5618.

44. Welch, M. D., DePace, A. H., Verma, S., Iwamatsu, A., and Mitchison, T. J. (1997). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. *J. Cell Biol.* **138,** 375–384.

45. Miki, H., Sasaki, T., Takai, Y., and Takenawa, T. (1998). Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* **391,** 93–96.

46. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97,** 221–231.

47. Leung, T., Chen, X. Q., Tan, I., Manser, E., and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol. Cell. Biol.* **18,** 130–140.

48. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367,** 40–46.

49. Sanders, L. C., Matsumara, F., Bokoch, G. M., and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* **283,** 2083–2085.

50. Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J., and Kumar, R. (2000). Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. *J. Biol. Chem.* **275,** 12041–12050.

51. Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M., and Schwartz, M. A. (1999). A role for p21-activated kinase in endothelial cell migration. *J. Cell Biol.* **147,** 831–843.

52. Sells, M. A., Boyd, J. T., and Chernoff, J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145,** 837–849.

53. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393,** 805–809.

54. Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393,** 809–812.

55. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeleton dynamics. *Nat. Cell Biol.* **1,** 253–259.

56. Manser, E., Loo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol. Cell* **1,** 183–192.

57. Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L., and Cerione, R. A. (1998). A novel regulator of p21-activated kinases. *J. Biol. Chem.* **273,** 23633–23636.

58. Zhao, Z., Manser, E., Loo, T. H., and Lim, L. (2000). Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. *Mol. Cell. Biol.* **20,** 6354–6363.

59. Kuroda, S., Fukata, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A., and Kaibuchi, K. (1996). Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1. *J. Biol. Chem.* **271,** 23363–23367.

60. Erickson, J. W., Cerione, R. A., and Hart, M. J. (1997). Identification of an actin cytoskeletal complex that includes IQGAP and the Cdc42 GTPase. *J. Biol. Chem.* **272,** 24443–24447.

61. Fukata, M., Kuroda, S., Fujii, K., Nakamura, T., Shoji, I., Matsuura, Y., Okawa, K., Iwamatsu, A., Kikuchi, A., and Kaibuchi, K. (1997). Regulation of cross-linking of actin filament by IQGAP1, a target for Cdc42. *J. Biol. Chem.* **272,** 29579–29583.

62. Kuroda, S., Fukata, M., Nakagawa, M., Fujii, K., Nakamura, T., Oobuko, T., Izawa, I., Nagase, T., Nomura, N., Tani, H., Shoji, I., Matsuura, Y., Yonehara, S., and Kaibuchi, K. (1998). Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin-mediated cell–cell adhesion. *Science* **281,** 832–835.

63. Qiu, R. G., Abo, A., and Steven Martin, G. (2000). A human homolog of the *C. elegans* polarity determinant Par-6 links Rac and Cdc42 to PKC$\zeta$ signaling and cell transformation. *Curr. Biol.* **10,** 697–707.

64. Joberty, G., Petersen, C., Gao, L., and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat. Cell Biol.* **2,** 531–539.

65. Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D., and Pawson, T. (2000). A mammalian PAR-3–PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat. Cell Biol.* **2,** 540–547.

66. Curran, S., and Murray, G. I. (1999). Matrix metalloproteinases in tumour invasion and metastasis. *J. Pathol.* **189,** 300–308.

67. Kheradmand, F., Werner, E., Tremble, P., Symons, M., and Werb, Z. (1998). Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. *Science* **280,** 898–902.

68. Malliri, A., Symons, M., Hennigan, R. F., Hurlstone, A. F., Lamb, R. F., Wheeler, T., and Ozanne, B. W. (1998). The transcription factor AP-1 is required for EGF-induced activa-

tion of Rho-like GTPases, cytoskeletal rearrangements, motility, and *in vitro* invasion of A431 cells. *J. Cell Biol.* **143,** 1087–1099.

69. Westermarck, J., and Kahari, V. M. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* **13,** 781–792.
70. Banyard, J., Anand-Apte, B., Symons, M., and Zetter, B. R. (2000). Motility and invasion are differentially modulated by Rho family GTPases. *Oncogene* **19,** 580–591.
71. Narumiya, S., and Morii, N. (1993). Rho gene products, botulinum C3 exoenzyme and cell adhesion. *Cell. Signalling* **5,** 9–19.
72. Zhong, C., Kinch, M. S., and Burridge, K. (1997). Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. *Mol. Biol. Cell* **8,** 2329–2344.
73. van Leeuwen, F. N., Kain, H. E., van der Kammen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology: Opposing roles for the small GTPases Rac and Rho. *J. Cell Biol.* **139,** 797–807.
74. Sander, E. E., and Collard, J. G. (1999). Rho-like GTPases: Their role in epithelial cell–cell adhesion and invasion. *Eur. J. Cancer* **35,** 1905–1911.
75. Evers, E. E., Zondag, G. C., Malliri, A., Price, L. S., ten Klooster, J. P., van der Kammen, R. A., and Collard, J. G. (2000). Rho family proteins in cell adhesion and cell migration. *Eur. J. Cancer* **36,** 1269–1274.
76. O'Connor, K. L., Nguyen, B. K., and Mercurio, A. M. (2000). RhoA function in lamellae formation and migration is regulated by the $\alpha 6\beta 4$ integrin and cAMP metabolism. *J. Cell Biol.* **148,** 253–258.
77. del Peso, L., Hernandez-Alcoceba, R., Embade, N., Carnero, A., Esteve, P., Paje, C., and Lacal, J. C. (1997). Rho proteins induce metastatic properties *in vivo*. *Oncogene* **15,** 3047–3057.
78. Yoshioka, K., Matsumura, F., Akedo, H., and Itoh, K. (1998). Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J. Biol. Chem.* **273,** 5146–5154.
79. Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T., and Narumiya, S. (1999). An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nat. Med.* **5,** 221–225.
80. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000). Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406,** 532–535.
81. Leung, T., Manser, E., Tan, L., and Lim, L. (1995). A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* **270,** 29051–29054.
82. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* **15,** 1885–1893.
83. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* **15,** 2208–2216.
84. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997). p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16,** 3044–3056.
85. Wasserman, S. (1998). FH proteins as cytoskeletal organizers. *Trends Cell Biol.* **8,** 111–115.
86. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389,** 990–994.
87. Leung, T., Chen, X. Q., Manser, E., and Lim, L. (1996). The p160 RhoA-binding kinase ROK$\alpha$ is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol.* **16,** 5313–5327.
88. Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y., and Narumiya, S. (1997). p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.* **404,** 118–124.
89. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T., and Narumiya, S. (1999). Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat. Cell Biol.* **1,** 136–143.
90. Nakano, K., Takaishi, K., Kodama, A., Mammoto, A., Shiozaki, H., Monden, M., and Takai, Y. (1999). Distinct actions and cooperative roles of ROCK and mDia in Rho small G protein-induced reorganization of the actin cytoskeleton in Madin–Darby canine kidney cells. *Mol. Biol. Cell* **10,** 2481–2491.
91. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273,** 245–248.
92. Kawano, Y., Fukata, Y., Oshiro, N., Amano, M., Nakamura, T., Ito, M., Matsumura, F., Inagaki, M., and Kaibuchi, K. (1999). Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase *in vivo*. *J. Cell Biol.* **147,** 1023–1038.
93. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* **271,** 20246–20249.
94. Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K., and Park, M. (2000). Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Mol. Biol. Cell* **11,** 1709–1725.
95. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* **285,** 895–898.
96. Kimura, K., Fukata, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1998). Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. *J. Biol. Chem.* **273,** 5542–5548.
97. Fukata, Y., Oshiro, N., Kinoshita, N., Kawano, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., and Kaibuchi, K. (1999). Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* **145,** 347–361.
98. Gardner, K., and Bennett, V. (1987). Modulation of spectrin–actin assembly by erythrocyte adducin. *Nature* **328,** 359–362.
99. Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., and Tsukita, S. (1998). Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/

moesin (ERM) proteins and regulates their head-to-tail association. *J. Cell Biol.* **140,** 647–657.

100. Nakano, K., Takaishi, K., Kodama, A., Mammoto, A., Shiozaki, H., Monden, M., and Takai, Y. (1999). Distinct actions and cooperative roles of ROCK and mDia in Rho small G protein-induced reorganization of the actin cytoskeleton in Madin–Darby canine kidney cells. *Mol. Biol. Cell* **10,** 2481–2491.
101. Matsui, T., Yonemura, S., Tsukita, S., and Tsukita, S. (1999). Activation of ERM proteins *in vivo* by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases. *Curr. Biol.* **9,** 1259–1262.
102. Lamb, R. F., Roy, C., Diefenbach, T. J., Vinters, H. V., Johnson, M. W., Jay, D. G., and Hall, A. (2000). The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. *Nat. Cell Biol.* **2,** 281–287.
103. Tominaga, T., Ishizaki, T., Narumiya, S., and Barber, D. L. (1998). p160ROCK mediates RhoA activation of Na-H exchange. *EMBO J.* **17,** 4712–4722.
104. Oude Weernink, P. A., Schulte, P., Guo, Y., Wetzel, J., Amano, M., Kaibuchi, K., Haverland, S., Voss, M., Schmidt, M., Mayr, G. W., and Jakobs, K. H. (2000). Stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho-kinase. *J. Biol. Chem.* **275,** 10168–10174.
105. Toker, A. (1998). The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* **10,** 254–261.
106. Gilmore, A. P., and Burridge, K. (1996). Molecular mechanisms for focal adhesion assembly through regulation of protein–protein interactions. *Structure* **4,** 647–651.
107. Genda, T., Sakamoto, M., Ichida, T., Asakura, H., Kojiro, M., Narumiya, S., and Hirohashi, S. (1999). Cell motility mediated by Rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. *Hepatology* **30,** 1027–1036.
108. Shore, P., and Sharrocks, A. D. (1995). The MADS-box family of transcription factors. *Eur. J. Biochem.* **229,** 1–13.
109. Treisman, R., Alberts, A. S., and Sahai, E. (1998). Regulation of SRF activity by Rho family GTPases. *Cold Spring Harbor Symp. Quant. Biol.* **63,** 643–651.
110. Hill, C. S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81,** 1159–1170.
111. Carnac, G., Primig, M., Kitzmann, M., Chafey, P., Tuil, D., Lamb, N., and Fernandez, A. (1998). RhoA GTPase and serum response factor control selectively the expression of MyoD without affecting Myf5 in mouse myoblasts. *Mol. Biol. Cell* **9,** 1891–1902.
112. Wei, L., Zhou, W., Croissant, J. D., Johansen, F. E., Prywes, R., Balasubramanyam, A., and Schwartz, R. J. (1998). RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. *J. Biol. Chem.* **273,** 30287–30294.
113. Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98,** 159–169.
114. Mullins, R. D., Heuser, J. A., and Pollard, T. D. (1998). The interaction of Arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA* **95,** 6181–6186.
115. Machesky, L. M., and Insall, R. H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8,** 1347–1356.
116. Obermeier, A., Ahmed, S., Manser, E., Yen, S. C., Hall, C., and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. *EMBO J.* **17,** 4328–4339.
117. Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S., and Alberts, A. S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Mol. Cell* **5,** 13–25.

Received August 29, 2000

# Identification of Rac-regulated genes using cDNA - representational difference analysis (cDNA-RDA) in combination with microarraying

*Arndt A. P. Schmitz, Robert Lucito, and Linda Van Aelst**

Cold Spring Harbor Laboratory

1 Bungtown Road

P. O. Box 100

Cold Spring Harbor

NY 11724, USA

* corresponding author
Phone: 1 (516) 367-6829
Fax: 1 (516) 367-8815
Email: vanaelst@cshl.org

# 1. INTRODUCTION

Small GTPases of the Ras superfamily are molecular switches which cycle between an active GTP-bound and an inactive GDP-bound state. They integrate signals from the cell surface to the nucleus, regulating important cellular activities. For example, Ras itself is activated when extracellular growth factors such as PDGF or EGF bind to their receptors at the cell surface. This activation of Ras leads ultimately to changes in the transcriptional activity of the cell, e.g. via the canonical MAPK cascade. Constitutively activated, mutant forms of Ras such as RasV12 are found frequently in human tumors and it is widely assumed that this oncogene acts via transcriptional activation of growth and proliferation pathways.

While the Rho family members, including Rho, Rac, and Cdc42, are best known for control of the actin cytoskeleton, they have also been linked to transcriptional activation. For example, activation of Rac triggers the activation of p38 and JNK MAPKs, as well as NFκB pathways [1]. Furthermore, numerous studies support a role for Rac in proliferation, invasion, and the control of cell adhesion. All these events occur over a longer time scale compared to the short-term changes of the actin cytoskeleton, and it is well assumed that these events require transcriptional activation. However, while pathways leading from Rac to the nucleus have been identified, information on genes regulated by Rac (or other members of the Rho GTPase family) remains scarce.

Within the last decade, several methods have been developed to identify changes in gene expression. These include serial analysis of gene expression (SAGE) [2], differential display (DD) [3, 4], representational difference analysis of cDNAs (cDNA-RDA) [5, 6], and suppression subtractive hybridization (SSH) [7, 8]. Noteworthy, each of the above techniques has its limitations. For example, Harris et al. identified differentially expressed genes in Aflatoxin B1 - treated hepatocytes, using in parallel DD, cDNA-RDA, and SSH, and each of these three methods found a small, non-overlapping set of differentially expressed genes [9]. Even more recently, expression analysis utilizing microarray technology has become available [10]. This technique depends on the availability of reliable cDNA clones that can be arrayed, whereas the former methods allow the identification of novel sequences in incompletely

characterized organisms. One major advantage of cDNA-RDA compared to other methods is its low level of false positives, because RDA eliminates those fragments which are present in both DNA populations. Furthermore, cDNA-RDA does not require sophisticated equipment.

We recently used cDNA-RDA to identify genes differentially expressed between cell lines induced and not induced for expression of the small GTPase Rac under the control of an inducible promoter. cDNA-RDA is a modified form of RDA, a PCR-based differential cloning method (Fig. 1) [11, 12]. In this technique, one DNA population (called the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester. RDA relies on the use of representations of the DNAs of interest. In brief, a representation is prepared by restriction digestion of the DNA (e.g. *Dpn*II in the case of cDNA-RDA), ligation of adaptor oligonucleotides, and subsequent PCR amplification.

One major task is the analysis of the difference product resulting from the cDNA-RDA experiment (Fig. 1c). To identify the most promising candidate genes, we and others [13] used the microarray technique as detailed below (Fig. 2). Only the clones with the highest differential expression as judged by microarraying were sequenced and further pursued. By doing so, we were able to increase significantly the number of clones screened per cDNA-RDA experiment.

## 2. MATERIALS

### 2.1. Equipment

1. A PCR machine capable of handling 0.5 ml PCR tubes to do cDNA-RDA (e.g. Perkin Elmer DNA Thermal Cycler 480).
2. A PCR machine which can hold 96-well plates (e.g. Perkin Elmer GeneAmp 9600) or up to four 96-well plates simultaneously (e.g. MJ Research PTC-225) to process the samples for microarraying.
3. A speedvac (e.g. Savant DNA 100).

4. 8-channel pipetters such as Labsystems Finnpipette 4510000 (0.5 - 10 μl) and 4510020 (5 - 50 μl) and a repeat pipette (e.g. Brinkmann Eppendorf 22260006) are very helpful for working with 96-well plates.
5. 96-well PCR plates are obtained from Perkin Elmer (N801-0560), 96-well plates for dilutions of DNA samples are retrieved from Nunc, while 96-well plates for microarraying (with V-shaped bottom) are obtained from Corning-Costar. Plates are covered either with caps (during PCR, Perkin Elmer N801-0535) or sealing film (Sigma Z36,968-3).
6. UV crosslinker (Stratalinker 2400, Stratagene).
7. Minifold I dot – blotting apparatus (Schleicher and Schuell SRC096/0).
8. Hybond-N+ nylon membrane (Amersham Pharmacia Biotech RPN203B).
9. Microcon YM-30 ultrafiltration columns (Amicon) to purify and concentrate labeling reactions.
10. A vacuum oven.
11. A microarrayer, e.g. Cartesian PixSys 5500 (Cartesian Technologies, Irvine, CA).
12. Pins for the arrayer (Chipmaker 2, Telechem International).
13. Silanated glass slides (Corning).
14. A humidified hybridization chamber (Telechem International).
15. A scanner suitable for microarray fluorescence detection, such as GSI Lumonics ScanArray3000 or Axon GenePix4000.
16. Analysis software: We made use of ScanAlyze (Stanford University) or Axon GenePix to determine features and for the quantitative analysis of the resulting TIFF files.

### 2.2. Enzymes and reagents

1. Organic solvents such as 70 % and 100 % ethanol (EtOH), isopropanol (iPrOH), chloroform, dimethylsulfoxide (DMSO), and phenol / chloroform / isoamylalcohol (25 / 24 / 1) saturated with TE pH 8.0 (Sigma P 2069).
2. FastTrack mRNA isolation kit (Invitrogen K1593-02).
3. CopyKit cDNA synthesis kit (Invitrogen L1311-03).
4. *Dpn*II restriction enzyme and 10 x *Dpn*II buffer (New England Biolabs 543L).

5. 10 mg / ml tRNA (Sigma R 8759) is used as a carrier during precipitations of small amounts of DNA.
6. Sheared salmon sperm DNA (Stratagene 201 190, diluted to 50 ng / $\mu$l).
7. DNA molecular weight marker $\phi$X174 *Hae*III digest (New England Biolabs 302-6L).
8. T4 ligase and 10 x T4 buffer (New England Biolabs 202S).
9. The primers listed in Table 1.
10. AmpliTaq polymerase, 25 mM $MgCl_2$, and 10 x Taq buffer without $MgCl_2$ (5 U / $\mu$l, Perkin Elmer N8080-153).
11. dNTPs (100 mM, Roche Molecular Biochemicals 1 696 064).
12. Mung Bean Nuclease (MBN) and 10 x MBN buffer (New England Biolabs 250S).
13. Qiaquick gel extraction kit (Qiagen 28704).
14. *Bam*HI restriction enzyme, 10 x *Bam*HI buffer, and 10 mg / ml BSA (New England Biolabs 136S).
15. Calf intestinal phosphatase (CIP) and 10 x CIP buffer (New England Biolabs 290S).
16. Qiaquick PCR purification kit (Qiagen 28104).
17. DNA polymerase I Klenow fragment and 10 x reaction buffer (Amersham Pharmacia Biotech E2141).
18. Cy3-dCTP and Cy5-dCTP for probe labeling (Amersham Pharmacia Biotech PA53021 and PA55021).
19. dRhodamine dye terminator kit for non-radioactive DNA sequencing (Perkin Elmer 403045).

## 2.3. Buffers

1. 3 M sodium acetate (NaAc), pH 5.2.
2. Elution buffer (EB; 10 mM Tris/HCl pH 8.5).
3. 6 x GLB (Gel Loading Buffer, 30 % glycerol and 0.25 % bromophenol blue. Not necessary to autoclave.)
4. 500 mM Tris/HCl pH 8.9 (It is important to use Tris base adjusted to pH with HCl instead of Tris Hydrochloride adjusted to pH with NaOH.)

5. 100 mM $MgCl_2$.
6. 200 mM $(NH_4)_2SO_4$, sterile filtered.
7. TE Buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).
8. 10 M ammonium acetate ($NH_4Ac$).
9. EE Buffer (30 mM EPPS (Sigma E 1894) pH 8.0, 3 mM EDTA pH 8.0), sterile filtered.
10. 5 M NaCl.

### 2.4. PCR Buffers

PCR buffers are prepared from autoclaved or sterile filtered stock solutions and autoclaved water (Note 1). All PCR buffers are made immediately before use and stored on ice.

1. PCR buffer 1 is used in Subheading 3.2.1.3. for the preparation of representations. This buffer consists of 67 mM Tris pH 8.9, 4 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mg/ml BSA, 16 mM $(NH_4)_2SO_4$, 320 $\mu$M each dNTP, 1.25 $\mu$M primer RBgl24, and 0.04 U / $\mu$l (15 U / tube) Taq.
2. PCR buffer 2 is identical to buffer 1, except that the primer and the Taq polymerase are added later. This buffer is used for the first PCR in each round of cDNA-RDA described in Subheadings 3.2.2.3. and 3.2.3., step 4.
3. PCR buffer 3 is also identical to buffer 1, except that primer NBgl24 (in Subheading 3.2.2.4.) or IBlg24 (in Subheading 3.2.3., step 4) are used. This buffer is employed during the second PCR in each round of cDNA-RDA.
4. PCR buffer 4 is used for the single colony PCR described in Subheading 3.3.3. This buffer consists of 12 % DMSO in 1 x Taq buffer supplemented with 2 mM $MgCl_2$, 200 $\mu$M each dNTP, 0.5 $\mu$M primer SP6R, 0.5 $\mu$M primer T7E, and 0.025 U / $\mu$l (0.625 U / well) Taq polymerase.
5. PCR buffer 5 is a standard PCR buffer used in Subheading 3.3.5. for reamplification. Buffer 5 is identical to buffer 4, except that the DMSO is omitted.

## 3. METHODS

A detailed description of the different steps in cDNA-RDA and the analysis of the difference product by microarraying is given. More common techniques used throughout our study, such as Western, Northern, and Southern blot analysis, are not described here in detail.

### 3.1. Preparation of cDNAs

In order to obtain meaningful results by cDNA-RDA, the samples used for the preparation of tester and driver need to be selected carefully. It is best to differ in only one parameter and to compare two samples matched as close as possible. For this reason, we made use of an inducible system for the expression of the target gene [14-16].

1. Perform time courses and titrate the concentration of inducer to establish optimal induction conditions for the selected inducible clones.
2. Grow approximately 5 x 10 ^ 7 cells of one selected clone in one incubator. Split cells into two halves and induce one half of the population. For example, in the case of MDCK cells with expression of RacV12 under the control of the tetracycline-off system, two days in the absence of doxocycline were sufficient to induce expression of RacV12 to the level of endogenous Rac [17]. Continue to use the same batch of medium for tester and driver populations. Harvest approximately 1 x 10 ^ 8 each of uninduced and induced cells by scraping into ice-cold PBS. Process immediately or store the cell pellet at -80 °C. Before continuing, check by Northern or Western blot analysis to see whether induction was successful.
3. Isolate mRNA from cells induced and not induced for expression of the gene of interest. Several commercial kits are available. We used the Fast Track kit for mRNA isolation and the CopyKit for cDNA preparation (both from Invitrogen). A yield of approximately 3 $\mu$g mRNA / 10 ^ 7 cells can be expected, depending on the cell type. Use 5 $\mu$g mRNA to generate approximately 7 $\mu$g cDNA (note 2).

### 3.2. cDNA-RDA

#### 3.2.1. Preparation of Representations

Representations are obtained by digestion of the cDNA sample of interest with a frequently cutting restriction enzyme such as *Dpn*II (GATC), followed by ligation to short

adaptor oligonucleotides and amplification by PCR, using the same sequences as primers. Representations to be compared to each other by cDNA-RDA have to be prepared simultaneously.

3.2.1.1. Digestions of the cDNAs

1. Digest the two cDNA samples derived from the induced and the uninduced cells in parallel. Each digest consists of 1.2 μg of cDNA in 10 μl 10 x *Dpn*II buffer, 10 μl 10 U / μl *Dpn*II, and $H_2O$ to a total volume of 100 μl overnight (16 h) at 37 °C (note 3).
2. Add 1 μl 10 μg / μl tRNA, vortex, and extract twice with 100 μl phenol/chloroform. Add 10 μl 3M NaAc pH 5.2 and 330 μl 100 % EtOH (kept at - 20 °C), vortex, and incubate at - 70 °C for 10 min.
3. Spin 10 min at 14 k rpm at 4 °C, and wash the pellets with 500 μl 70 % EtOH (kept at -20 °C). Spin again, remove wash solutions as completely as possible by pipetting. Dry in a speedvac at medium heat and resuspend carefully in 12 μl EB (note 4).
4. Transfer 2 μl to a tube containing 8 μl $H_2O$ and 2 μl 6 x GLB. Load the samples onto a 2 % agarose gel, side-to-side with 200 ng undigested cDNA (to check digest), a dilution series of 50 - 100 - 200 ng sheared salmon sperm DNA (to check recovery and concentration), and 200 ng of ϕX174 *Hae*III as the marker.

3.2.1.2. Ligation

The ligation is preceded by an incubation step ensuring optimal conditions for the adaptors to hybridize to the cDNA fragments as follows:

1. To the remaining 10 μl of each cDNA digest, add 7.5 μl 62 μM RBgl24, 7.5 μl 62 μM RBgl12, 3 μl of 10 x T4 buffer, and 2 μl $H_2O$.
2. Mix well and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55 °C.
3. Unplug the heatblock, transfer the block with the two tubes to the cold room, and allow the temperature to decrease to 10 - 15 °C over the next 90 min (check).
4. Add 1 μl T4 ligase to each tube, mix by pipetting, and incubate for 16 h at 12 °C.

3.2.1.3. Amplification by PCR

To ensure equal conditions during preparation of representations, these PCRs must be performed in parallel in the same PCR machine using the same mastermix for both sets of reactions.

1. Add 970 µl of TE supplemented with 200 µg/ml (20 µl of 10 µg / µl) tRNA to each of the two ligations, vortex, and store on ice.
2. Label two times ten 0.5 ml PCR tubes, a set each for the induced and the uninduced sample, respectively. Prepare 8 ml of PCR buffer 1 and aliquot 360 µl into each of the 20 PCR tubes. Add 40 µl from each of the two diluted ligations to the ten corresponding tubes. Vortex each tube briefly before adding one drop mineral oil to each tube.
3. Cycle all 20 tubes together 20 times in a two-temperature PCR cycle (1 min at 95 °C and 3 min at 72 °C). Finish the reaction by a final extension at 72 °C for 10 min.
4. To check the outcome of the PCR, run 8 µl of the reaction from each tube + 1.6 µl 6 x GLB on a 2 % agarose gel, using ϕX174 *Hae*III as the marker and 200 - 300 - 400 ng sheared salmon sperm DNA to check yield (approximately 300 ng per lane can be expected). All ten aliquots prepared from the induced sample should look identical. Also, all ten aliquots from the uninduced sample should look identical among themselves. Often, a band pattern specific for each of the two samples is observed.
5. Withdraw the PCR reactions under the mineral oil and collect each of the two samples (in total 4 ml from the ten matching tubes) in a 15 ml Falcon tube. Extract both pooled samples twice with 3 ml phenol/chloroform. Add 400 µl 3M NaAc and 8 ml iPrOH. Incubate for 15 min at 4 °C (or overnight at - 20 °C), and spin 30 min at 4 °C.
6. Wash pellets once with 5 ml 70 % EtOH (kept at - 20 °C), recentrifuge for 5 min, remove supernatants, and dry pellets in a desiccator. Redissolve pellets in 500 µl TE. Check recovery by diluting 2 µl sample 1:10 with TE and running 2, 5, and 10 µl on a 2 % agarose gel with 100 - 250 - 500 ng salmon DNA as standards.
7. Estimate the amount of DNA on the gel and use it to calculate the total amount of representation recovered. Approximately 150 µg for each of the two representations

prepared can be expected. This corresponds to ca. a 400-fold amplification, since the representations were made from 0.4 μg template each (note 5).

3.2.1.4. Removal of the adaptors by digestion

Before the representations can be used in a cDNA-RDA reaction, the adaptors at their ends must be cleaved by restriction enzyme digestion.

1. Use 100 μg of each of the two representations prepared in Subheading 3.2.1.3. for a digest to remove adaptors. In a 2 ml eppendorf tube, add 100 μg representation to a reaction mix consisting of 100 μl 10 x *Dpn*II buffer, 100 μl 10 U / μl *Dpn*II, and water to 1 ml final volume. Incubate overnight at 37 °C, preferentially in an incubator.
2. Extract twice with 1 ml phenol/chloroform. Add 100 μl 3M NaAc and 1 ml iPrOH. Incubate for 15 min at 4 °C (or overnight at - 20 °C), and spin the two tubes at 14 K rpm at 4 °C for 15 min. Wash pellets once with 1 ml 70 % EtOH (kept at - 20 °C), and carefully dry pellets in a speedvac. Redissolve carefully in 100 μl TE by pipetting and vortexing.
3. Check recovery and completeness of digestion by preparing 10 μl of a 1:10 dilution and run 1, 2.5, and 5 μl samples on a 2 % agarose gel. Include 200 ng samples of the undigested representations (Subheading 3.2.1.3.). The band pattern before and after digestion should be identical, except that after digestion it will be shifted slightly downward due to the removal of 48 bp of adaptor sequence. Use 100 - 250 - 500 ng salmon DNA to check yield. Adjust concentrations to 500 ng / μl with TE, pipet, and revortex.

3.2.2. First round of cDNA-RDA

Since cDNA-RDA identifies only samples upregulated in one sample relative to the other, we perform two cDNA-RDA experiments in parallel (see Fig. 1). To identify downregulated genes, add the representation derived from induced cells as the driver to the tester derived from uninduced cells. To identify upregulated genes, use the representation derived from uninduced cells as the driver, and add it to the tester derived from induced cells. In this experiment, it is

advisable to add the induced gene of interest back to the driver, since this sequence may otherwise constitute a major part of the difference product (see note 6).

3.2.2.1. Ligation of new adaptors to tester

1. Set up in parallel two ligations to obtain testers from each of the two samples. Each ligation contains 2 $\mu$l of 500 ng / $\mu$l digested representation (see Subheading 3.2.1.4.), 7.5 $\mu$l 62 $\mu$M NBgl24, 7.5 $\mu$l 62 $\mu$M NBgl12, 3 $\mu$l 10 x T4 buffer, and 10 $\mu$l $H_2O$.
2. Mix well, and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55 °C. Unplug the heatblock and transfer the block with the tubes to the cold room and allow the temperature to fall to 10 - 15 °C during the next 90 min.
3. Add 1 $\mu$l T4 Ligase, mix by pipetting, and incubate for 16 h at 12 °C.
4. Add 70 $\mu$l TE supplemented with 20 $\mu$g / ml tRNA, and store on ice.

3.2.2.2. Hybridization

1. Adjust one thermoblock with glycerol or mineral oil filled holes to 67 °C and another one to 98 °C.
2. For each of the two reactions, add 80 $\mu$l of 500 ng / $\mu$l (40 $\mu$g) digested representation, corresponding to the driver (see Subheading 3.2.1.4.), to 40 $\mu$l of 10 ng / $\mu$l (0.4 $\mu$g) representation ligated to new adaptors (see Subheading 3.2.2.1.; the tester). Vortex the eppendorf tubes and extract once with 120 $\mu$l phenol / chloroform. Add 30 $\mu$l 10 M $NH_4Ac$, vortex, and add 380 $\mu$l EtOH (kept at - 20 °C). Incubate at - 70 °C for 10 min, warm the samples for 2 min at 37 °C, and spin at 4 °C at 14 K rpm for 20 min. Wash twice with 500 $\mu$l 70 % EtOH, and dry carefully in a speedvac (up to 2 min, without heating).
3. Resuspend each pellet in 5 $\mu$l EE by pipetting and vortexing four times for 30 sec each. Spin down very briefly and carefully add 35 $\mu$l mineral oil. Denature DNA at 98 °C for 4 min, add 1.5 $\mu$l 5 M NaCl to the aqueous phase by pricking through the mineral oil with the pipett tip, and incubate at 67 °C for 20 h.

3.2.2.3. First PCR

1. Remove mineral oil from the hybridizations (see Subheading 3.2.2.2.). Add tRNA (8 µl 5 µg / µl) to the hybridization mix. Mix by pipetting, add 390 µl TE, and vortex.
2. Prepare 1.5 ml of PCR buffer 2, distribute 352 µl each into four PCR tubes. Add 40 µl (4 µg total DNA) from the one hybridization to two of the four tubes and 40 µl from the other hybridization to the remaining two tubes, and place them in a PCR machine kept at 72 °C.
3. Add 3 µl 5 U / µl Taq polymerase to each of the four tubes, vortex, and incubate for 5 min at 72 °C, during which time the 3' ends of the adaptor sequences will be filled in. Add 10 µl of 62 µM primer NBgl24 to each tube, vortex, add mineral oil, and cycle 10 times in a two-temperature PCR (keep at 95 °C for 1 min and at 72 °C for 3 min).

3.2.2.4. MBN digest and second PCR

The PCR step described in Subheading 3.2.2.3. selectively amplifies the sequences enriched in the tester. To improve amplification of tester-specific sequences during the second PCR amplification, the single stranded driver DNA is digested by MBN treatment.

1. Place a waterbath at 30 °C and a heat block at 98 °C.
2. Pool the identical samples obtained after the first PCR (Subheading 3.2.2.3.). Extract once with 600 µl phenol/chloroform and once with 600 µl chloroform. In a 2 ml eppendorf tube, add 80 µl 3M NaAc and 1 ml iPrOH.
3. Incubate for 1 h at - 20 °C, spin 15 min at 14 K rpm at 4 °C. Wash pellets once with 500 µl 70 % EtOH (kept at - 20 °C), recentrifuge for 5 min, and remove supernatants.
4. Dry pellets in a speedvac and redissolve carefully in 40 µl EB by pipetting and vortexing. Prepare 50 µl 2 x MBN buffer.
5. Incubate 20 µl from each of the two samples in parallel with 20 µl 2 x MBN buffer and 2 µl 10 U / µl MBN enzyme for 30 min at 30 °C.
6. Neutralize with 160 µl 50 mM Tris pH 8.9 and inactivate the MBN for 5 min at 98 °C, then store on ice.
7. Prepare 1.5 ml of PCR buffer 3, distribute 360 µl into four PCR tubes. Add 40 µl from each sample from the preceding step to two PCR tubes. Do not combine the samples at

this step. Vortex, add mineral oil, and perform 20 PCR cycles of 1 min at 95 °C and 3 min at 72 °C.

8. To evaluate the outcome of the PCR, run 10 µl of the reaction from each tube + 2 µl 6 x GLB on a 2 % agarose gel and use 100 - 200 - 300 ng sheared salmon sperm DNA to check yield (a yield of approximately 200 ng / 10 µl can be expected). In the case of a low yield, supplement each tube with 3 µl of fresh Taq polymerase and perform three additional PCR cycles.
9. Pool the identical samples and extract them once with 600 µl phenol/chloroform and once with 600 µl chloroform. In a 2 ml eppendorf tube, add 80 µl 3M NaAc and 1 ml iPrOH. Incubate overnight at - 20 °C.
10. Centrifuge for 15 min, 14 K rpm at 4 °C. Wash pellet once with 500 µl 70 % EtOH (kept at - 20 °C), and dry the pellets in a speedvac.
11. Redissolve the pellets carefully in 100 µl TE by pipetting and vortexing. Prepare 20 µl of a 1:5 dilution and run 2.5, 5, and 10 µl on a 2 % agarose gel. Include lanes with 100 - 200 - 300 - 400 ng salmon DNA to check yield (approximately 20 µg / each sample is to be expected). Adjust the concentration to 100 ng / µl and store the difference product 1 (DP1) at - 20 °C.

3.2.3. Second round of cDNA-RDA

The second round of cDNA-RDA is basically a repetition of the first round, incorporating the following changes: The product of the first round of cDNA-RDA (DP1) is used as the tester, while the driver is still the digested representation. The driver to tester ratio is increased from 100:1 to 800:1. Also, a new set of primers is used.

1. Remove the NBlg24 adaptors from 5 µg of DP1 with *Dpn*II following the protocol provided in Subheading 3.2.1.4. Resuspend after precipitation in 50 µl TE. Check the yield by agarose gel electrophoresis and adjust the concentration to 20 ng / µl with TE.
2. Ligate new adaptors to 100 ng (5 µl) of digested DP1, using the same conditions and concentrations of reagents as described in 3.2.2.1., but using IBgl24 and IBgl12 instead

of NBgl24 and NBgl12. Incubate overnight, add 50 µl TE supplemented with 20 µg / ml tRNA, and store on ice.

3. Mix 40 µl (50 ng) of tester just ligated to new IBgl adaptors and 80 µl of 500 ng / µl (40 µg) driver (prepared in Subheading 3.2.1.4.). Hybridize, following the instructions given in Subheading 3.2.2.2.
4. Perform first PCR, MBN digest, and second PCR as outlined in Subheading 3.2.2.3. and Subheading 3.2.2.4., substituting primer IBgl24 for NBgl24. Adjust the concentration to 100 ng / µl and store the difference product 2 (DP2) at - 20 °C (note 7).

### 3.3. Analysis of the obtained difference products

#### 3.3.1. Southern Blot analysis

After completion of the cDNA-RDA experiment (which will take approximately 2 to 3 weeks), the difference products (DP2) are analyzed. A first standard control is performed by Southern Blot analysis, using the induced gene (e.g. Rac) as a positive control to probe equal amounts of each driver and tester representation, DP1, and DP2 transferred to a nylon membrane. If looking for genes downregulated by Rac, the hybridization signal from the Rac probe should be stronger in the driver than in the tester and absent from both DP1 and DP2. If looking for genes upregulated by Rac, the hybridization signal should be stronger in the tester than in the driver and more stronger in both DP1 and DP2 compared to tester. This signal is likely to be detected, although to a lesser extent, even when the induced gene was added back to the driver (see Note 6).

If the blot indicates that the cDNA-RDA experiment was successful, the individual clones contained in the DP2 are analyzed. The DP2 library is subcloned, individual inserts are amplified and revalidated by microarraying in order to sequence only the most promising candidate genes.

#### 3.3.2. Subcloning

1. Digest 1 µg DP2 (see Subheading 3.2.3.) with *Dpn*II as described in Subheading 3.2.1.4.

2. After redissolving in 20 µl EB, add 4 µl 6 x GLB and run at low voltage on a 2 % agarose gel ca. 4 cm distance from the wells. With a fresh scalpel, cut out the whole lane from 800 – 100 bp, leaving the cleaved adaptors behind.
3. Use the Qiaquick gel extraction kit to recover the DNA and ligate the adaptor-free DP2 to a bacterial expression vector (e.g. pGEM7Zf(-)) digested with *Bam*HI and dephosphorylated with CIP) as described in Subheadings 3.2.1.2. and 3.2.2.1. Transform into highly competent *E. coli* and plate the library.

3.3.3. Single colony PCR

Individual clones are directly amplified from the bacterial colonies by using the bacteria as the template in a PCR reaction with primers directed against the sequences surrounding the multiple cloning site of the vector. We suggest to process four 96 well plates from each DP2 library.

1. Prepare 2.75 ml of PCR buffer 4 (note 8). Pipette 325 µl into each well of the first column of a 96-well PCR plate. With an 8-channel pipetter, distribute 25 µl into each of the wells across the plate.
2. Pick a single white colony from the plate using a sterile pipet tip, and dip the tip into a well of the PCR plate. Repeat 94 times, inoculating each well with an individual white colony picked at random using fresh tips. Include one well as a negative control without any template.
3. Amplify by PCR using a three temperature program (5 min at 94 °C for initial denaturation; 25 cycles of 1 min each at 94 °C, 60 °C, and 72 °C; additional 10 min at 72 °C for final elongation and hold at 4 °C). Run 5 µl sample, 5 µl $H_2O$, and 2 µl 6 x GLB on a 2 % agarose gel with ϕX174 *Hae*III as the standard, and photograph. Store remainder of the PCR reactions at - 20 °C.

3.3.4. Exclusion of cDNA clones which are identical to the induced gene of interest

It is to be expected that the induced gene (e.g. Rac) will be found as a difference product if the tester is derived from induced cells, even, as mentioned above, when the induced gene was

added back to the driver (see also note 6). To prevent sequencing these clones, we identified them by dot-blotting experiments.

1. Prepare a 96-well plate with 10 µl TE in each well. Add 1 µl of each of the PCR reactions from Subheading 3.3.3. into the corresponding wells.
2. Following the manufacturer's instructions, place a Hybond-N+ nylon membrane in the minifold I dot-blotting apparatus with and apply vacuum. Load your DNA samples in the corresponding wells. Fix the DNA to the membrane, e.g. by UV-crosslinking.
3. Prepare 50 ng of radiolabeled double-stranded DNA probe from your induced gene (e.g. full length Rac1) and hybridize to the dot-blot filter.

3.3.5. Re-amplification for microarraying

The amplified clones need to be re-amplified in order to obtain sufficient amounts for microarraying as well as to ensure that the amplification was specific.

1. Prepare 10 ml of PCR buffer 5. Add 100 µl each to the wells of a 96-well PCR plate. With an 8-channel pipetter, add 1 µl each from the PCR reactions (see Subheading 3.3.3.) into the corresponding well. Amplify by PCR using the following program: 1 min at 94 °C for initial denaturation; 25 cycles of 1 min each at 94 °C, 60 °C, and 72 °C; additional 10 min at 72 °C for final elongation; hold at 4 °C).
2. Run 5 µl of each of the PCR products and 1 µl 6 x GLB on a 2 % agarose gel with ϕX174 *Hae*III as the standard, and photograph. Transfer the samples to a Corning 96-well plate, supplement with 10 µl 3 M NaAc and 200 µl EtOH, and keep for 16 h at -20 °C.
3. Centrifuge for 20 min at + 4 °C and 3750 rpm. Aspirate supernatants, wash with 100 µl 70 % EtOH, spin for 5 min, and aspirate supernatants. Dry the pellets in a vacuum oven at maximal 50 °C, resuspend in 15 µl of 3 x SSC per well, and store at -20 °C.

3.3.6. Microarraying

In our microarraying experiments, the clones derived from a cDNA-RDA experiment are arrayed on a chip. Representations from each driver and tester are labeled with a fluorophore, mixed, and then hybridized simultaneously to the arrayed candidate genes.

3.3.6.1. Array preparation

1. Array the re-amplified inserts from Subheading 3.3.5. on commercially prepared silanated slides, using a Cartesian PixSys 5500 or equivalent.
2. Place the array in a humidified chamber for 3-5 min to hydrate the spots. Crosslink the slide by UV irradiation with 60 mjoules in a UV crosslinker. Rehydrate the slide in the humidified chamber and snap dry by heating on the surface of a hot plate for several seconds. Wash the chip in 0.1 % SDS for approximately 10 sec, in deionized water for ca. 10 sec, and then denature the chip in boiling MilliQ water for ca. 1 - 2 min. After denaturation, immediately immerse the array in ice-cold benzene-free ethanol for several seconds. Take out and allow the chip to dry. Perform the wash procedure from the SDS to the ice-cold ethanol also with the cover slips to be used with the arrays.

3.3.6.2. Sample preparation

1. In parallel, denature 10 $\mu$g each of the representations derived from induced and uninduced cells in the presence of 5 $\mu$g of a random nonamer primer in a total of 100 $\mu$l $H_2O$. Use either representations ligated to adaptors (see Subheading 3.2.1.3.) or with the adaptors cleaved off (see Subheading 3.2.1.4.).
2. To each sample, add 12 $\mu$l of 10 x Klenow buffer and supplement with 33$\mu$M dNTPs, 10 nmoles of either Cy3 or Cy5, and 4 units of Klenow fragment. Incubate the reactions at 37 °C for 2 h.
3. Combine the two reactions and remove unincorporated nucleotides by centrifugation through a Microcon YM-30 ultracentrifugation column according to the manufacturer's instructions. Adjust the eluate containing the labeled sample to a volume of 15 $\mu$l and a concentration of 3 x SSC and 0.2 % SDS, and denature by heating to 95 °C for 5 min.
4. Carefully place the 15 $\mu$l of labeled representations on the array prepared in Subheading 3.3.6.1. and slowly place a cover slip on the array. Insert into hybridization chamber according to manufacturer's instructions and incubate in the dark at 67 °C overnight.
5. After the overnight hybridization, disassemble the hybridization chamber and wash the array at room temperature in 0.1 % SDS, 0.2 x SSC for 90 sec, followed by a 30 sec wash in 0.2 x SSC and a final 30 sec wash in 0.05 x SSC.

6. After washing, arrays are imaged in a scanner. Images are saved as TIF files and feature definition and quantitative analysis are performed with either ScanAlyze or Axon GenePix. This analysis yields a text file that is imported into Microsoft Excel or Access for further analysis.

3.3.7. Non-radioactive sequencing of PCR products

Sequence all clones which are found to be differentially expressed based on microarraying (e.g., fluorescence ratio greater than 2). However, do not sequence the Rac clones already identified by dot-blotting in Subheading 3.3.4.

1. To obtain sufficient material for non-radioactive sequencing, repeat the PCR from Subheading 3.3.5. with only the selected clones as the templates. These PCR samples are checked on a gel and then purified using the Qiaquick PCR purification kit. Determine the concentration of the samples by $OD_{260}$, which should be at least 25 ng / $\mu$l.
2. Perform the sequencing reactions using 8 $\mu$l rhodamine dye terminator kit, 2 $\mu$l 1.6 $\mu$M primer SP6R or T7E, 250 ng ds DNA PCR product, and $H_2O$ to 20 $\mu$l. Submit the obtained sequences to BLAST searches.

3.4. Further characterization of candidate genes

It is advisable to reconfirm the expression pattern for these genes which showed altered expression in the microarray experiments by an independent method, such as Northern blot analysis. In the case of less abundant genes, quantitative RT-PCR might be required.

Further analysis of interesting clones will be dependent on the nature of the gene. To further obtain information on the Rac-induced genes, we started to explore the effects of various growth factors and drugs on the transcription of these genes. We used reagents known to activate or to interfere with previously characterized signaling pathways of Rac. For example, the transcription levels of candidate genes can be compared between resting cells and cells stimulated with PDGF or EGF. Furthermore, Rac is known to activate e.g. the p38 MAP kinases and a drug, SB202190, is known as a selective inhibitor of these kinases [18]. The transcription of candidate genes can be compared between cells induced for Rac and either left untreated or treated with SB202190. These experiments are also performed using the

microarray technique, allowing to monitor simultaneously the effect of these reagents on the genes identified by cDNA-RDA.

## 4. NOTES

1. A major challenge in cDNA-RDA experiments is the high risk of contamination, since cDNA-RDA is able to amplify very small differences between two representations. Hence, while performing cDNA-RDA, the use of gloves, sterile filter tips, single use individually wrapped pipettes, and sterile single use plasticware is highly recommended. Be aware that not all types of plastic withstand the phenol/chloroform solutions used. Therefore, use rather polypropylene than polystyrene tubes. Also, all reagents are used only for cDNA-RDA.
2. To prevent losing sequences later during preparation of the representations (Subheading 3.2.1.), one can design specific primers for the preparation of cDNAs. Short or unusually composed mRNA sequences might contain either none or only one *Dpn*II site. To avoid their loss, use primers with a *Dpn*II site added at the 5' end instead of standard random or oligodT primers.

   Using large amounts of cells derived from an inducible expression system might not be possible in all cases. When starting with smaller amounts of cells (10^6), the Oligotex kit (Qiagen) is recommended for mRNA isolation. If working with even smaller amounts of defined starting material or tissue samples, it may be necessary to use specialized methods during the preparation of representations, such as discussed elsewhere [12, 6, 19]. When the samples to be used as tester or driver need to be pooled (e.g. progenitor cells of healthy individuals), it is advisable not to pool the cells or the mRNAs, but to prepare in parallel a representation from each individually and then to combine them.
3. Incubate in an incubator rather than in a waterbath or a thermoblock. An incubator is preferred as such to avoid evaporation of the solution and recondensation on the inner surface of the lid.

4. Be careful not to lose minute amounts of dried DNA in handling. This is a major source of low yields in the following PCR amplification steps. It is also important not to over-dry the DNA pellets, since they become more difficult to redissolve.
5. It is essential to reproducibly obtain representations before starting cDNA-RDA. If a typical yield of ca. 150 $\mu$g from 0.4 $\mu$g template is not obtained, this step has to be optimized (such as by preparing new buffers, new cDNAs and / or mRNAs).
6. Depending on the level of induction, the induced gene itself may become a major part of the difference product, when the induced sample is employed as the tester to identify upregulated genes. This can be suppressed by adding this particular transcript back to the driver during both rounds of cDNA-RDA. While others have recommended to substitute up to 50 % of the driver by the gene [6], we would advise to be more cautious and rather to substitute ca. 10 % of the driver. An optimal ratio can also be determined experimentally by titration. The Southern blot analysis described in Subheading 3.3.1. might not be quantitative enough to detect whether the addition had an effect on the composition of the DP2. However, the difference should be notable in the percentage of individual clones identified by dot-blotting (in Subheading 3.3.4.) as being the induced gene.
7. There are only a few ways to judge the quality of the cDNA-RDA experiment during the procedure. It is essential, however, that the yields and amplification rates described throughout the protocol are achieved. Also, digests should result in downward shifts of band patterns in agarose gels (as in Subheading 3.2.1.4., step 3). Finally, equal aliquots of driver and tester representations, DP1, and DP2 can be run together on a gel (to perform the Southern blot analysis described in Subheading 3.3.1.). The observation of appearing or disappearing bands from representation to DP1 and DP2 may indicate that the cDNA-RDA has worked. However, a constant band pattern does not necessarily indicate that the cDNA-RDA has failed. Best is to proceed with the Southern Blot analysis and use its outcome as described in Subheading 3.3.1. to judge the quality of the cDNA-RDA experiment.

8. We found that inclusion of 12 % DMSO in this PCR buffer significantly increased the yield, while addition of Triton X-100 had no effect on the yield and reduced the clarity of the run on an agarose gel.

Acknowledgments

The authors would like to thank Joan Alexander for sequencing and Chet Cunha (Friend's Academy H.S., Glen Cove, NY) for technical assistance.

This research was supported by grants from the U.S. Army and the NIH to Linda Van Aelst, who is also a Kimmel Foundation and Foundation V award recipient. Arndt A. P. Schmitz is in part supported by a post-doctoral fellowship from the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany).

References


1. Van Aelst, L., and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295-2322.
2. Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995) Serial analysis of gene expression. *Science* **270**, 484-487.
3. Liang, P., and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.
4. Liang, P., and Pardee, A.B. (Eds.) (1997) *Differential Display Methods and Protocols.* Methods Mol. Biol., Vol. 85. Humana Press, Totowa, NJ.
5. Hubank, M., and Schatz, D.G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**, 5640-5648.
6. Hubank, M., and Schatz, D.G. (1999) cDNA representational difference analysis: a sensitive and flexible method for identification of differentially expressed genes. *Methods Enzymol.* **303**, 325-349.
7. Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., and Siebert, P.D. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6025-6030.
8. Zuber, J., Tchernitsa, O.I., Hinzmann, B., Schmitz, A.C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schaefer, R. (2000) A genome-wide survey of RAS transformation targets. *Nature Genet.* **24**, 144-152.
9. Harris, A.J., Shaddock, J.G., Manjanatha, M.G., Lisenbey, J.A., and Casciano, D.A. (1998) Identification of differentially expressed genes in aflatoxin B1-treated cultured primary rat hepatocytes and Fischer 344 rats. *Carcinogenesis* **19**, 1451-1458.
10. Phimister, B. (Ed.) (1999) The Chipping Forecast. *Nature Genet.* **21** (Suppl.), 1-60.
11. Lisitsyn, N., Lisitsyn, N., and Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* **25**, 946-951.

12. Lisitsyn, N., and Wigler, M. (1995) Representational difference analysis in detection of genetic lesions in cancer. *Methods Enzymol.* **254**, 291-304.

13. Welford, S.M., Gregg, J., Chen, E., Garrison, D., Sorensen, P.H., Denny, C.T., and Nelson, S.F. (1998) Detection of differentially expressed genes in primary tumor tissues using representational differences analysis coupled to microarray hybridization. *Nucleic Acids Res.* **26**, 3059-3065.

14. Clackson, T. (1997) Controlling mammalian gene expression with small molecules. *Curr. Opin. Chem. Biol.* **1**, 210-218.

15. No, D., Yao, T.P., and Evans, R.M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3346-3351.

16. Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547-5551.

17. Jou, T.S., and Nelson, W.J. (1998) Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J. Cell Biol.* **142**, 85-100.

18. Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., and Young, P.R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739-746.

19. Lockhardt, D.J., and Winzeler, E.A. (2000) Genomics, gene expression, and DNA arrays. *Nature* **405**, 827-836.

FIGURE LEGENDS

**Fig. 1: Principle of cDNA-RDA. A.** Flowchart of cDNA-RDA. A. From cells either induced or left uninduced for expression of the gene of interest, representations are prepared by isolation of mRNAs, synthesis of cDNAs, restriction digest with *Dpn*II, ligation to adaptors, and amplification by PCR. The adaptors are subsequently removed by digestion in order to use the representation as the driver in a cDNA-RDA experiment. To use the representation as the tester, the adaptors are cut off and replaced by newly ligated adaptors of different sequence. Then, the tester from one sample is hybridized to an excess of driver from the other sample and sequences enriched in the tester are selectively amplified by PCR to obtain the difference product one (DP1). Finally, the DP1 is used as the tester in a new round of hybridization and amplification to result in difference product two (DP2). Note that by performing two sets of reactions in parallel, using cells from each of the two samples once as tester and once as driver against the other sample, up- as well as down-regulated genes can be identified. **B.** The three possible outcomes of the tester/driver hybridization. If a sequence is unique to the tester or present at a higher molar ratio in the tester than in the driver, it will be exponentially amplified. If a sequence is found in both driver and tester to equal amounts, only the strand from the tester population bears the adaptor and the sequence will be linearly amplified. If the sequence is found only in the driver, neither strand contains the adaptor sequence and the sequence will not be amplified. **C.** A typical difference product after two rounds of cDNA-RDA visualized by agarose gel electrophoresis. A difference product consists of a series of visible bands superimposed on a 'smear'. As indicated, each band may contain fragments of several genes whose different sizes can not be resolved on an agarose gel. Furthermore, candidate genes may be contained in the 'smear'. Finally, different fragments of the same gene can occur at different positions in the gel, since cDNA-RDA is based on digested cDNA.

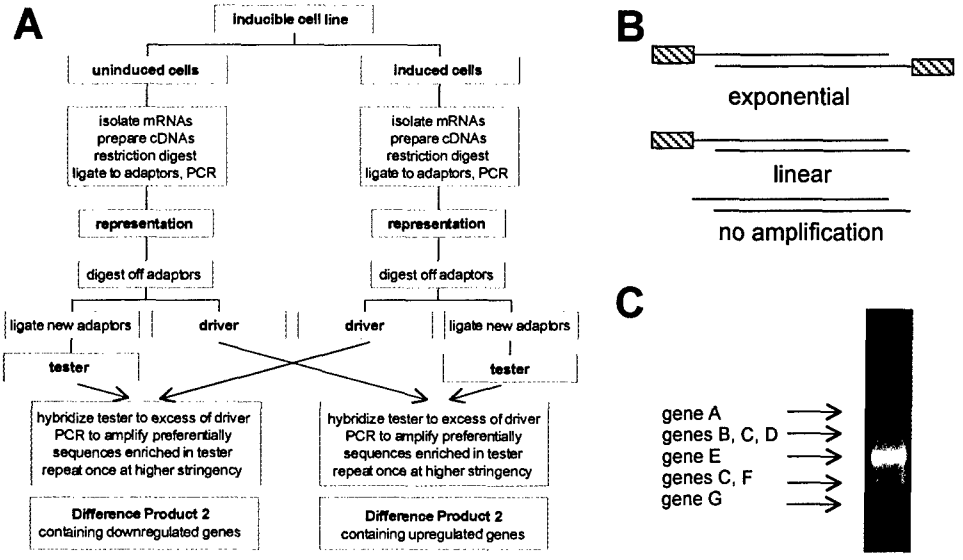
**Fig. 2: Analysis of cDNA-RDA products by the microarray technique.** 190 clones derived from a cDNA-RDA experiment using cells induced for RacV12 expression as the

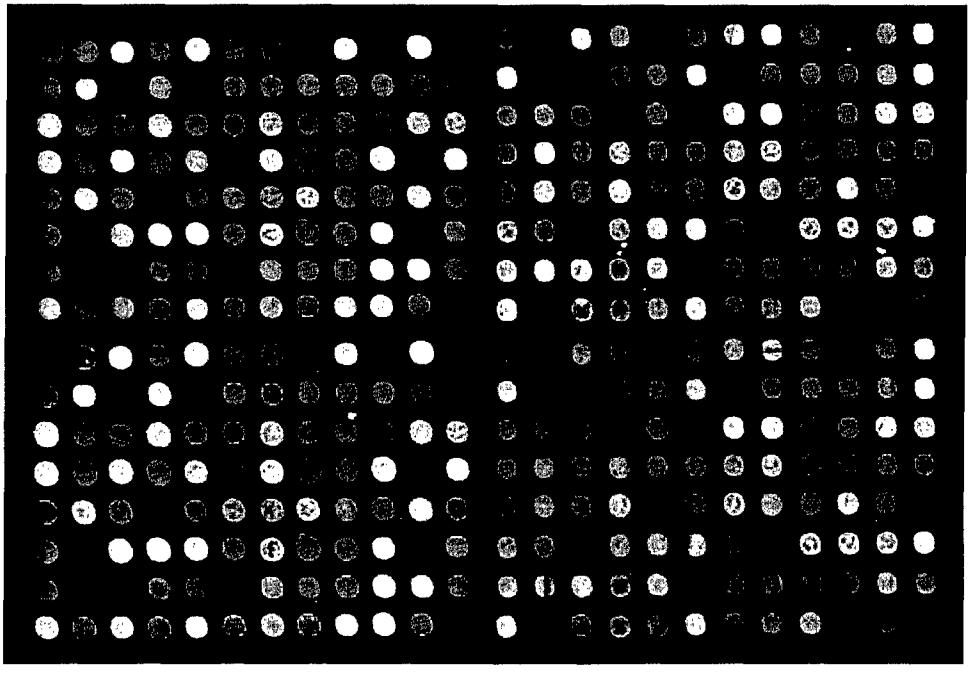
tester and uninduced cells as the driver were arrayed in duplicate. The array was then hybridized simultaneously to a representation from RacV12 induced cells (labeled in green) and to a representation from uninduced cells (labeled in red). The clones with different shades of green represent genes whose expression is upregulated as a result of RacV12 expression, whereas 'yellow clones' are transcripts unaffected by RacV12 expression. Genes downregulated by RacV12 would be identified as red spots, but are absent as expected from the design of this particular experiment. Such, microarraying allows rapid screening of a difference product for the clones with the highest differential expression ratio which are then further pursued.

*Table 1: Sequences of the used Primers.*

| Name | Sequence | Use | Ref. |
|---|---|---|---|
| RBgl24 | AGCACTCTCCAGCCTCTCACCGCA | Representation | 11 |
| RBgl12 | GATCTGCGGTGA | Representation | 11 |
| NBgl24 | AGGCAACTGTGCTATCCGAGGGAA | 1st round of cDNA-RDA | 11 |
| NBgl12 | GATCTTCCCTCG | 1st round of cDNA-RDA | 11 |
| IBgl24 | TCAGCATCGAGACTGAACGCAGCA | 2nd round of cDNA-RDA | a) |
| IBgl12 | GATCTGCTGCGT | 2nd round of cDNA-RDA | a) |
| SP6R | GGTGACACTATAGAATACTCAAGC | Single colony PCR | b) |
| T7E | TGTAATACGACTCACTATAGGGC | Single colony PCR | b) |
| Random nonamer | NNNNNNNNN | Probe labeling | a) |

Footnotes to Table 1: a) Robert Lucito, unpublished results; b) this report.


A
inducible cell line
uninduced cells
induced cells
isolate mRNAs
prepare cDNAs
restriction digest
ligate to adaptors, PCR
isolate mRNAs
prepare cDNAs
restriction digest
ligate to adaptors, PCR
representation
representation
digest off adaptors
digest off adaptors
ligate new adaptors
driver
driver
ligate new adaptors
tester
tester
hybridize tester to excess of driver
PCR to amplify preferentially
sequences enriched in tester
repeat once at higher stringency
hybridize tester to excess of driver
PCR to amplify preferentially
sequences enriched in tester
repeat once at higher stringency
Difference Product 2
containing downregulated genes
Difference Product 2
containing upregulated genes
B
exponential
linear
no amplification
C
gene A
genes B, C, D
gene E
genes C, F
gene G



Review

# THE ROLE OF RHO-GTPases IN DISEASE DEVELOPMENT

Benjamin Boettner and Linda Van Aelst*

Cold Spring Harbor Laboratories,

1 Bungtown Road, Cold Spring Harbor, NY 11724

*corresponding author; E-mail address: vanaelst@cshl.org

## Abstract

The functionality and efficacy of Rho GTPase signaling is pivotal for a plethora of biological processes. Due to the integral nature of these molecules, the dysregulation of their activities can result in diverse aberrant phenotypes. Dysregulation can, as will be described below, be based on an altered signaling strength on the level of a specific regulator or that of the respective GTPase itself. Alternatively, effector pathways emanating from a specific Rho GTPase may be under- or overactivated. In this review, we address the role of the Rho-type GTPases as a subfamily of the Ras-superfamily of small GTP-binding proteins in the development of various disease phenotypes. The steadily growing list of genetic alterations that specifically impinge on proper Rho GTPase function corresponds to pathological categories such as cancer progression, mental disabilities and a group of quite diverse and unrelated disorders. We will provide an overview of disease-rendering mutations in genes that have been positively correlated with Rho GTPase signaling and will discuss the cellular and molecular mechanisms that may be affected by them.

## 1. Introduction

The family of Rho GTPases comprise a large subfamily of the Ras-superfamily of GTPases. Among all Rho GTPases, Rac1 (Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42) and RhoA (Ras homologous member A) have been studied most extensively. Through the work of many laboratories the role, that Rho GTPases play in cellular processes as diverse as polarization, cell-cell and cell-matrix adhesion, membrane trafficking, cytoskeletal and transcriptional regulation and cell proliferation has made them a group of crucial regulators with a very general relevance (comprehensively reviewed in Hall, 1998; Van Aelst and D'Souza-Schorey, 1997).

As is the case for small GTPases in general, Rho GTPases are guanine nucleotide binding proteins, which cycle between an active GTP-bound and an inactive GDP-bound state, and are subject to distinct control mechanisms. In the inactive state, Rho GTPases are associated with a class of negative regulators, the Rho GDP dissociation inhibitors (GDIs), that stabilize the GDP-bound form of the GTPase and sequester them in the cytoplasm. Their active state is promoted by positive regulators called GDP/GTP exchange factors (GEFs) that (a) tether a given GTPase to a distinct subcellular location and (b) by virtue of their signature tandem Dbl homology (DH)/pleckstrin homology (PH) domain exchange GDP moieties associated with the inactive GTPases for GTP. As a consequence, a conformational switch is induced. This in turn renders the GTPase active and allows it to initiate a productive signaling complex with one of several effector proteins. This instigates an information flow to different cellular destinations via different molecular pathways with different physiological outcomes. The active GTP-bound state is counteracted by negative regulators, the GTPase activating proteins (GAPs), that catalyse the intrinsic ability of a small GTPase to hydrolyse the bound GTP-moiety to GDP (hence the name guanosine tri-phosphatases). Thus, effector binding is reversed and signaling activity halted, causing the biochemical system to come full circle. Understanding this biochemical basis for the function of GTPases has greatly benefited research and lead to the development of constitutively active (GTPase-deficient) and dominant negative (nucleotide exchange-defective) mutants that lock a respective GTPase in the GTP- or GDP-bound state. The introduction of such mutants into diverse experimental systems allows for either overactivation or functional deletion of a specific GTPase.

There is a growing list of disease-causing mutations in genes that have been associated with Rho GTPase signaling by means of functional prediction or insights obtained by direct biochemical analysis. These include GEFs, GAPs and effector proteins that appear to be part of quite diverse signaling networks. Surprisingly, though, only a single genetic lesion in a gene encoding a Rho GTPase itself, namely the RhoH gene, has been described thus far (see below). Other mutations that may inactivate a Rho gene or lead to an overactive version of the resulting protein due to a lack of extensive screening or functional redundancy have either escaped detection or simply are lethal. This latter possibility is underscored by the fact, that mouse embryos whose Rac1 or Cdc42 genes have been deleted by gene-targeted mutation die early in development (Sugihara et al., 1998; Chen et al., 2000). It may also reflect the multifunctional nature of Rho GTPases.

Loss-of-function or constitutive gain-of-function mutations in many Rho GTPases thus may interfere with a number of different cellular processes. Based on our current understanding and dependent on the precise physiological circumstances and cell-types under investigation, a single Rho GTPase can affect a diverse array of phenomena implicated in a cell's specific biology. In addition, there is also continued speculation that Rho-type GTPases need to cycle between their active and inactive states in order to exert their complete physiological potential (discussed by (Symons and Settleman, 2000)).

On the other hand, it is likely that regulators and effectors of Rho GTPases are expressed and act in a more specific manner, be it in the context of a specific cell-type, tissue-type or developmental process. Genetic loss-of-function mutations in these regulators or effectors, even in form of a germline mutation, may result in a weaker impairment than loss of the respective GTPase itself. The continuing revelation of novel genetic lesions in genes encoding Rho regulators and effectors fully supports this possibility.

The following sections summarize examples of disease processes whose underlying genetic alterations affect the normal function and regulation of Rho GTPases. We examine the importance of such mutations in cancer progression, mental disabilities and other disorders.

## 2. Rho GTPases in cancer progression

The evidence that directly implicates aberrant Rho-signaling activity in cancer has been obtained either by means of mutations uncovered in various genes encoding Rho-signaling components, or by screening and interference protocols that focus on specific aspects of cancer biology. For a detailed summary of the biological understanding of the pivotal role of Rho-type GTPases in cancer-related processes such as cell-proliferation, migration, invasion and metastasis, we refer the reader to some excellent recent reviews (Symons, 1996; Schmitz et al., 2000; Price and Collard, 2001; Pruitt and Der, 2001).

### *1.1. Rho GTPases in transformation*

In contrast to the "classical" oncogenic Ras proteins, such as N-Ras and K-Ras, that are frequently mutated in human cancers (Bos, 1988), to date only a single sequence alteration has been detected in a gene encoding a Rho GTPase. In a set of patients diagnosed with non-Hodgkin's lymphoma a t(3;4)(q27;p11-13) translocation was found to be responsible for the pathogenic progression of the disease. Upon closer examination of this locus, a genetic fusion of a gene encoding the newly designated RhoH/TTF GTPase with the LAZ3/BCL3 gene was detected. While a single distinct transcript of the fused loci could be amplified by RT-PCR, it remains elusive whether the promotion of the leukemic aberration is in some way attributable to the RhoH portion of the fusion product (Preudhomme et al., 2000). Targeted deletion of a closely related GTPase gene, namely that encoding RhoB in mice, significantly facilitated tumor development (Liu et al., 2001).

Despite RhoH being the only example of a Rho-specific mutation in humans thus far, it has been clearly demonstrated that Rho-family members play an important role in Ras-induced transformation. Evidence for this has been provided by experiments utilising constitutively active (GTPase defective) and dominant negative (nucleotide exchange defective) mutant forms of Rho GTPases in focus forming assays and their ability to enable growth in soft agar as well as tumor formation in nude mice. These protocols allow growth factor-independent proliferation and contact-independent growth to be assessed. Different laboratories have shown that the activation of distinct Rho GTPases is an essential step towards the fully transformed phenotype triggered by an activated Ras oncogene. Specifically, a dominant negative form of Rac1 markedly inhibits the focus forming activity invoked by Ras in fibroblasts but can not interfere with an activated, membrane-targeted version of the Raf-kinase which acts as the Ras-effector enhancing MAPK activity. In contrast, an activated form of Rac1 significantly enhances the focus forming potential of membrane-targeted Raf (Qiu et al., 1995a). These observations led to the conclusion that Rac is activated by Ras via a Raf-independent pathway. Further studies corroborated these findings and identified RhoA as a second Rho-family GTPase. Khosravi-Far *et al.* and Qiu *et al.* observed that the focus forming activity of a weakly transforming Raf-1 mutant was greatly enhanced when co-introduced together with constitutively active forms of Rac1 or RhoA into fibroblasts. In contrast, the dominant negative forms of Rac1 and RhoA could partially block Ras transformation (Khosravi-Far et al., 1995; Qiu et al., 1995b). In another study, Symons and co-workers added Cdc42 to the list of participants in that they demonstrated that Cdc42 enabled Ras-transformed cells to grow independent of anchorage (Qiu et al., 1997). Additional studies involving Cdc42 suggest that the ability of a GTPase to cycle could be an essential requirement for its transforming potential. In elegant experiments, Lin et al. introduced an F28L mutation into Cdc42, which subjects the GTPase to spontaneous GTP-binding coupled to a wild-type GTP-hydrolysis rate. Cycling in this mutant is therefore greatly accelerated. When stably expressed in fibroblasts, $Cdc42^{F28L}$ yields features of transformed cells including a reduced serum-dependency and anchorage-independency (Lin et al., 1997). Taken together, the genetic interactions summarized above led to the view that Ras not only triggers the activities of the Raf-MAPKK-MAPK cascade but also acts through an alternative route that stimulates Rho-type GTPases which in turn contribute as ancillary factors to the fully transformed phenotype. Furthermore, a picture emerged in which each of the investigated Rho GTPases appears to contribute to a different aspect of the transformed phenotype.

In contrast to the failure to localize mutations in Rho GTPase genes, there is a growing literature reporting transcriptionally upregulated levels of particular Rho proteins in many types of cancers including those as genetically diverse as those occurring in colon, breast, lung and pancreas (Suwa et al., 1998; Fritz et al., 1999; Clark et al., 2000; Mira et al., 2000; Schnelzer et al., 2000; van Golen et al., 2000; Kamai et al., 2001). Recent genomic analysis, combining a protocol selecting for highly metastatic melanoma cells in mice with microarray analysis identified the RhoC gene as a promotor of metastatic behaviour. Subsequently, directed expression studies verified this finding (Clark et al., 2000; van Golen et al., 2000). Whether this observation will hold true in human tissue systems as well, and what the concrete cellular consequences of this phenomenon are, await to be seen. It is noteworthy, however, that, in ductal adenocarcinomas of the

pancreas as well as in inflammatory breast cancer cells, RhoC expression was shown to be upregulated (Suwa et al., 1998; van Golen et al., 2000). An intriguing finding is that of the novel Cdc42-like GTPase Wrch-1 as a Wnt-1 transcriptional target (Tao et al., 2001). The Wnt signaling pathway, mostly through mutational analysis of the Wnt-family genes as well as the APC and β-catenin genes, has been implicated in tumorigenesis (Peifer and Polakis, 2000; Polakis, 2000). The contribution of Wrch-1 to the list of Wnt-responsive genes may help understand the mechanisms employed by Wnt-signaling to induce morphological changes, interfere with cell-cell adhesion and cell-extracellular matrix interactions and other phenomena. It also raises the interesting possibility of Rho GTPases functioning as a general class of mediators in multiple oncogenic pathways.

While all these changes in transcript and protein levels are likely to correlate with an elevation in signaling activity *in vivo*, there are few studies that directly assess the activation state of particular Rho GTPases under transforming conditions. One study that does address this issue is the one by Mira and colleagues who were able to monitor an increase in Rac3 activity in transformed and highly proliferative breast epithelial cells derived from human cancer samples (Mira et al., 2000).

### *2.1. Rho GTPase regulation by upstream activators*

As another line of evidence that implicates the Rho-family of GTPases in certain aspects of cancer development, members of the Dbl-family of Rho-GEFs have been classified as oncoproteins. Again, this classification is mainly based on the classical transformation assays mentioned above. The Dbl-family contains such well-investigated members as the founding member of the family, Dbl, the Vav, TIAM, Lcf proteins and others. They all share the tandem Dbl homology and PH homology motifs are now a hallmark feature for Rho-specific activator proteins. Distinct Dbl-family members may show either overlapping activity towards more than one of the Rho GTPases, or specifically activate only one of them. Their focus forming abilities are evoked by activating deletions and point mutations usually in regulatory regions of the molecules. For example, in the case of Dbl and Vav, the N-termini of the proto-oncoproteins have been found to repress their catalytic activities. Accordingly, deletion of these N-terminal sequences can relieve this repression and result in a constitutive activation of the GEF function. The structural basis for these autoinhibitory mechanisms and their release has been convincingly disclosed in an NMR spectroscopic analysis focusing on the Vav paradigm of Tyr-phosphorylation-mediated activation (Aghazadeh et al., 2000). In the case of Vav, stimulation of cells with particular extracellular agents ensues rapid and transient phosophorylation of Vav which in turn abolishes its autoinhibitory function with a concomitant increase in GEF-activity (for review, see (Bustelo, 2000).

Upon closer inspection of the available literature, we noticed that naturally occurring gain-of-function mutations in patient-derived material have so far only been described for Dbl and the more recently identified LARG and TIAM genes. The Dbl oncogene was originally isolated from a human diffuse B-cell lymphoma DNA in a coupled gene transfer/transformation assay (Srivastava et al., 1986), while LARG (Leukemia-associated Rho guanine exchange factor) was isolated as a fusion partner of the MLL (mixed lineage leukemia) gene in acute myeloid leukemia (Kourlas et al., 2000).

Subsequently, work by two groups produced biochemical evidence for LARG's identity as a Rho activator (Fukuhara et al., 2000; Reuther et al., 2001). The protein's tandem DH/PH domain appears to be specific for RhoA since LARG can activate the Rho-dependent serum response factor (SRF) but not Rac/Cdc42-dependent Jun kinase (JNK) signaling (Reuther et al., 2001). It is also possible, that the RGS (regulator of G-protein signaling) domain harbored by the protein serves to couple G-protein-coupled receptors (GPCRs) and heterotrimeric G proteins of the G alpha(12) family to Rho-dependent signaling (Fukuhara et al., 2000). These initial biochemical studies, together with the finding that LARG is expressed in all human tissues examined, will surely promote further interest in this molecule.

TIAM (T-cell invasion and metastasis gene) was found in an extensive screen, that was designed to isolate invasion-promoting genes in T-cells (Habets et al., 1994). Subsequently, TIAM was demonstrated to display Rac-specific exchange activity (Michiels et al., 1995). In light of earlier experiments that showed Rac to promote the spread of cancer cells and metastasis in nude mice (Qiu et al., 1995a), these data proposed a TIAM/Rac-dependent, invasion-promoting signaling pathway. This opened a different perspective on the role of Rho-proteins in cancer progression, namely their function in metastasis and invasion in aggressive cancer cells. This property of some Rho GTPases has since been reiterated in different experimental settings (e.g. the one described for the finding of RhoC above). In fact, analysis of the TIAM locus in a set of renal-cell carcinomas revealed several mutant TIAM alleles. One of these mutations, A441G, maps to the protein's N-terminal pleckstrin homology domain and could potentially interfere with the proper membrane localization and functional activity of the protein. In focus forming assays, the A441G mutation can convert TIAM into a transforming molecule which further underlines its relevance in malignant processes (Engers et al., 2000). Interestingly, a putative metastasis suppressor, nm23H1, which was isolated from murine melanomas by subtraction cloning associates with TIAM and seems to negatively regulate cell motility of tumor cells of murine and human origin (Leone et al., 1991; Kantor et al., 1993; MacDonald et al., 1996). This activity correlates with a downregulation of Rac1 (Otsuki et al., 2001). Thus nm32H1 may prove to be an interesting physiological link to TIAM and Rac regulation during invasion and metastasis. It has to be mentioned, that, depending on the specific cell type under investigation (Hordijk et al., 1997) and the particular ECM composition faced by those cells (Sander et al., 1998), TIAM can also generate an adhesion-promoting effect. In epithelial MDCK cells, its expression may even revers the loss of E-Cadherin mediated adhesion induced by oncogenic Ras (Hordijk et al., 1997). Therefore, the involvement and regulation of TIAM in invading and metastasizing cancer cells might be more complex than the current data suggest.

### *2.3. Downstream effectors and effector pathways of Rho GTPases*

What is the nature of the underlying signaling pathways mediating distinct aspects of cellular transformation triggered by Rho-GTPases and which are the immediate downstream effectors regulating them?

A necessary requirement for a cell to transit from a normal to a transformed state is the dysregulation of its cell cycle machinery. From earlier observations, it became evident that cyclin D1, as a key factor required for the G1/S transition whose levels oscillate throughout the cell cycle, is transcriptionally upregulated by the Ras-induced Raf/MEK/ERK pathway (Cheng et al., 1998; Kerkhoff and Rapp, 1998). Subsequently, it has been demonstrated that Rac can also stimulate activity at the cyclin D1 promotor (Westwick et al., 1997; Gille and Downward, 1999). Cyclin D1, as a functional consequence of its enhanced synthesis, teams up with its cognate cyclin-dependent kinase partners CDK4 and CDK6. The major substrate for the phosphorylation-acitvity of the complex is the Retinoblastoma protein whose subsequent degradation triggers progression through the G1-phase of the cell cycle (Sherr and Roberts, 1999). It is worth noting, that a survey of human breast cancer etiology in patients has revealed the cyclin D1 gene to be amplified in 20% of the cases examined (Dickson et al., 1995). Moreover, cyclin D1 protein levels appeared to be elevated in more than 50% of mammary carcinomas (Bartkova et al., 1994; Gillett et al., 1994; McIntosh et al., 1995). Further corroborating the relevance of this transcriptional target, Yu et al. observed that cyclin D1-deficient mice are able to resist cancer development induced by the Ras and Neu oncogenes (Yu et al., 2001). It is conceivable that Rac could either contribute to the full oncogenic upregulation of cyclin D1 transcription by Ras or that it leads to a sustained promotor activity. However, Rac can also activate the cyclin E1 gene in Ras-independent pathways. Consistent with the latter possibility, activation of integrin signaling has recently been demonstrated to modulate the levels of cyclin D1 protein in a Rac-dependent manner in order to trigger proliferation of primary cells in culture. Surprisingly, in this case Rac appears to influence cyclin D1 expression on the translational rather than the transcriptional level (Mettouchi et al., 2001). Apart from cyclin D1 as a transcriptional target, the expression of the cell-cycle regulator c-myc, induced by PDGF, has been demonstrated to rely on Rac activity and to occur independently of Ras. Together, this information suggests, that a single Rho GTPase might affect the cell cycle via a number of different pathways (Chiariello et al., 2001).

Rho, on the other hand, seems to impinge on another class of cell cycle regulatory proteins, namely the cyclin/CDK complex inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Evidence for this comes from the observations, that (1) Rho activity interferes with $p21^{Cip1}$ in that it suppresses its induction (Olson et al., 1998), and (2) $p27^{Kip1}$ degradation is facilitated by Rho (Weber et al., 1997; Hu et al., 1999). Hence, as in the case of cyclin D1 upregulation, these Rho-specific phenomena lead to a stabilization of cyclin/CDK activity which consequently is thought to accelerate progression through the cell cycle.

To date, it remains unclear which of the many effector molecules of the Rho-family GTPases unambiguously relay an oncogenic potential *in vivo* and which of the activated signaling pathways are the ones responsible. There have been no activity-modifying mutations described in any of the known Rho GTPase effector proteins in cancerous cells. One of the candidates relaying the Rac signal to the cell cycle machinery in order to achieve transformation, however, is the Ste20-like p21 PAK-kinase. PAK kinases were first identified by Manser et al. who performed biochemical overlay assays with the intention to isolate novel Rac1 and Cdc42 effector proteins (Manser et al., 1994). A catalytically inactive form of PAK can effectively suppress Ras-induced transformation in some cell lines (Tang et al., 1997) and, it has been shown that PAK activity correlates

with cyclin D1 promotor induction (Joyce et al., 1999). Similarly, the function of Rac3 in the proliferation of breast tumor cells appears to correlate with increased PAK activity (Mira et al., 2000). PAK apart from its correlation to cell proliferative events, also has been related to aspects of microfilament reorganization and invasiveness in breast cancer cells. In this setting, a kinase-dead version of PAK1 can suppress the motile and invasive phenotypes of otherwise highly invasive human MDA-MB435 cells (Adam et al., 2000). Although these data suggest a role for PAK in tumorigenesis/metastasis it remains unclear as to whether PAK is dependent on Rac-activity to exert its effect. It has been demonstrated that PAK-independent pathways emanating from Rac are likely to participate in promotion of Rac's transforming potential. It also has been demonstrated, that Rac-mutants engineered to no longer bind PAK, but that still tether other effectors, can persist to evoke transformation of fibroblasts (Joneson et al., 1996; Lamarche et al., 1996). This is suggestive of the idea, that the cell cycle machinery can be either targeted by Rac through a PAK-independent pathway or by multiple pathways in a given cell and/or that different pathways in different cell types operate to that end.

The ROCK effector-kinases have been proposed to convey Rho's transforming ability. ROCK-type kinases serve as regulators of the actomyosin network in that they promote phosphorylation of the regulatory light chain of myosin in a Rho-dependent manner. This in turn stimulates contractility of actin filaments which is the basis of cell motility. A constitutively active version of the kinase can synergize with activated Raf in conventional transformation assays (Sahai et al., 1999). An additional and intriguing piece of evidence for ROCK's role in tumorigenesis has been provided by Narumiya and colleagues who developed and successfully applied a ROCK-specific inhibitor (Y-27632). Rat MM1 hepatoma cells are able to migrate through a monolayer of mesothelial cells because of a mutationally enhaced motility. This migratory behaviour is enhanced by transfection with a dominant positive form of ROCK, while conversely, it is inhibited by a ROCK mutant acting as a dominant negative version of the kinase. Treatment with Y-27632 can block Rho-mediated invasion in MM1 cell cultures and, moreover, can interfere with the dissemination of MM1 cells when implanted into the peritoneal cavity of syngeneic rats (Itoh et al., 1999). In the first experiments with human cells , Somlyo et *al.* found that Y-27632 can inhibit human prostate cancer cells from disseminating when introduced into immune-compromised mice (Somlyo et al., 2000). Whether this inhibitor will prove to be genuinely ROCK-specific and whether the data from these animal experiments can be extrapolated to the invasion of human metastatic cells in human tissues has to be shown by further careful investigation.

Previously, it was found that ROCK-dependent phosphorylation of the Ezrin/Radixin/Moesin (ERM)-family member Ezrin is a necessary requirement in the transformation process induced by oncogenic Dbl-proteins. ERM-proteins are thought to link the actin cytoskeleton to membrane regions undergoing dynamic morphological changes. Contact inhibition that has been perturbed by Net or Dbl in fact can be re-established by the introduction of a ROCK-desensitized Ezrin mutant. Promisingly, ROCK-dependent Ezrin phosphorylation can be blocked by Y-27632 (Tran Quang et al., 2000). Ezrin also has been found to interact with Hamartin, the gene product of the TSC1 tumor suppressor gene locus (Lamb et al., 2000) as well as RhoGDI and the Dbl oncoprotein (Takahashi et al., 1997; Takahashi et al., 1998). Together, these insights

strongly emphasize the importance of Rho/ROCK mediated signaling in cancer progression.

A potentially oncogenic mechanism involving a Rho GTPase and impacting on vesicle trafficking has recently brought to light (Wu et al., 2000). Whereas Rho GTPases have been implicated in various steps of membrane trafficking (for review see (Ridley, 2001), the significance for this in the oncogenic processes remains ill-understood. Cdc42 has been implicated by Wu et al. in the functional modulation of the Golgi coatamer complex. The coatamer complex serves to shuttle cargo from the endoplasmic reticulum to the Golgi apparatus and Cdc42 directly associates with the γ-COP subunit (Wu et al., 2000). The same authors found that Cdc42 has to target γ-COP in order to generate the transformed phenotype characteristic of the 'fast cycling' $Cdc42^{F28L}$ mutant mentioned above. This observation provides the first clue for the involvement of membrane trafficking in the cellular transformation (Wu et al., 2000). It was known earlier, that Cdc42 localizes to the Golgi compartment and that it affects several transport steps such as the exit of apical and basolateral proteins from the trans-Golgi network as well as endocytic transport to the basolateral plasma membrane in polarized cells (Erickson et al., 1996; Kroschewski et al., 1999; Musch et al., 2001). While the precise consequences of the Cdc42/γ-COP interaction are not well understood, it may be a first molecular link for Cdc42's association with the Golgi compartment and its local effects therein.

Another door that just has been opened concerns the role of Rho GTPases in the establishment of cellular polarity. While the general relevance of Rho GTPases in these processes has been demonstrated, the discovery of the PAR6 protein as a Rac1 and Cdc42 effector contributes a first molecular insight. PAR6 is a constituent of the Par-6/Par-3/PKCζ complex that is vital for the establishment of cellular polarity in diverse systems [Joberty, 2000 #167; Johansson, 2000 #170; Lin, 2000 #168; Qiu, 2000 #138]. In mammalian cells, PAR3 and PAR6 are associated with tight junctional structures. Overexpression of PAR6 or a dominant active form of Cdc42 results in a disruption of tight junctional structures and Rac1/Cdc42 engagement of the complex is thought to induce PKCζ activity [Joberty, 2000 #167; Qiu, 2000 #138]. Moreover, ectopically expressed PAR6 enhances the transforming potential of Rac1 [Qiu, 2000 #138]. Since tight junctional integrity is required for the maintenance of cell polarity and cell polarity is abolished in transformed cells, targeting of the Par-6/Par-3/PKCζ complex by activated Rac1 or Cdc42 could contribute to malignant transformation. The activation of atypical PKC isoforms including the PKCζ previously has been correlated to the control of cell growth and survival. Ectopic amounts of PKCζ can counteract apoptotic signals and experimental down-regulation of PKCζ levels and activity impair cell proliferation and activation of the NF-κB transcription which in many situations prevents apoptosis (Berra et al., 1993; Dominguez et al., 1993; Diaz-Meco et al., 1996).

It has been demonstrated that the cell survivial machinery appears to be directly affected by Rho GTPases. Like oncogenic Ras (Finco et al., 1997; Mayo et al., 1997), Rac, Cdc42 and Rho positively regulate the transcription at NF-κB-dependent promotors (Sulciner et al., 1996; Perona et al., 1997) and thus may prevent cells driven to a transformed state from undergoing apoptosis. This pathway also appears to interfere with Ras-induced apoptosis (Joneson and Bar-Sagi, 1999) and the expression of dominant active Rac can prevent suspension-induced apoptosis ("anoikis") of epithelial cells (Coniglio et al., 2001) as well as apoptosis triggered by serum deprivation of fibroblastic

cells (Ruggieri et al., 2001). It will be interesting to see, whether PKCζ activation in polarity determining complexes by Rac1 and/or Cdc42 will connect through a distinct signaling pathway to antiapoptic and proliferative events like e.g. activation of NF-κB. Of note is the observation that Rac2-deficient cells derived from gene targeted mice display significantly reduced survival in the presence of growth factors as compared to control cells. This property, furthermore, has been correlated with a failure to induce the survival factors Akt and BAD/Bcl-XL (Yang et al., 2000). Targeted deletion and transgenic expression experiments in mice revealed a role also for Rho GTPase genes and their products in mediating apoptosis and cell survivial in a context-dependent manner (Cleverley et al., 2000; Costello et al., 2000; Liu et al., 2001). Rho B mutant mouse embryo fibroblasts (MEFs) when transformed with with the H-Ras and adenovirus E1A oncogenes displayed a significantly elevated resistance to apoptosis after being exposed to DNA-damaging reagents as compared to non-targeted cells (Liu et al., 2001). However, in hemopoetic cells Rho has clearly been correlated with cell survivial signaling (Costello et al., 2000). The thymocyte-specific lck promotor was used to drive the expression of the Rho-inhibitor C3 (bacterial toxin C3 transferase from Clostridium botulinum). C3 selectively ADP-ribosylates and inactivates RhoA, B and C (Boquet, 1999). Under these conditions, pre-T cells that have not undergone β selection to assemble vβ-chains into functional pre-T cell receptors (TCRs) are subject to massive apoptosis. This cell death phenomenon is abrogated in a p53 loss of function situation suggesting a p53-dependent mechanism that is governed by Rho-activity. After formation of functional pre-T cell receptors, however, Rho appears to promote survival via a p53-independent and BCL-2-sensitive pathway (Costello et al., 2000). Another study by Cleverley et al. shows that the same mice, when 4 to 8 months old, develop aggressive thymic lymphoblastic lymphomas (Cleverley et al., 2000). Analysis of the tumours revealed a lack of heterogeneity in the vβ-chain of the T-cell receptor (TCR) complex which led the authors to hypothesize a monoclonal origin of the malignant cells. Since lck-driven C3 expression alone does not ensue tumor formation per se, it is likely that an additional genetic alteration underlies the observed clonal expansion. Whether the same mechanism that suppresses apoptosis in RhoB nullizygous MEFs also is at work in T cells and how it is constituted will still need further investigation.

In summary, the significance of Rho GTPase signaling impinges on various aspects of oncogenesis. As exemplified in the aforementioned, cell cycle progression in tumor cells, their adhesive properties, migratory and invasive behaviour and escape from apoptotic extinction all seem to be affected by Rho GTPase activity. Whereas a scaffold emerges in which a particular Rho GTPase is linked to specific physiological effects, defined signaling pathways still need to be worked out. As is the case with Y-37632 as a ROCK inhibitor, other participants in specific signaling pathways may serve as drug targets to inhibit malignant processes that depend on Rho GTPase activity. In addition, given, that dysregulations provoked by Rho GTPases phenocopy cancerous aspects of oncogenesis in general, it is tempting to speculate that Rho signaling might be contributing to other oncogenic pathways as well. The identification of Wrch-1 as a transcriptional target of oncogenic Wnt signaling is a first example for such interdependencies.

## 3. An emerging role for Rho-GTPases in neurodegenerative disorders

Rho GTPases are currently gaining increasing attention for their involvement in a complex of heterogenous neuropathological disorders, namely nonsyndromic, X-chromosome linked forms of mental retardation (commonly referred to as MRX or XLMR). MRX affects approximately 1 in 500 males and represents about 25% of all genetically manifested cases of mental retardation. The only feature in individuals affected by MRX is an impairment of their cognitive functions. While no gross anatomical alterations in brain structures has been observed in MRX-inflicted individuals, closer histological inspection has revealed that the hippocampus and certain cerebellar ventricals often are increased in size. In contrast, the cerebral cortex often appears reduced in size when compared to unaffected control tissue (Reiss et al., 1991; Reiss et al., 1994). Detailed microscopic analysis showed, that the dendritic spines in the affected regions are thinner and more elongated in MRX-patients. Furthermore, the synaptic contacts they establish are more reminiscent of those made by immature spines (Rudelli et al., 1985; Hinton et al., 1991). Spine synapses are considered to relay the majority of functional excitatory synaptic communication. Moreover, they are regarded as the structures displaying most of the ongoing synaptic plasticity that determines the efficacy of 'learning and memory' processes (Matus, 1999).

Spine morphology is particularly dependent on actin structures and processes that continuously remodel them. These is a function that in many respects requires the directed regulation and activity of the Rho-family of GTPases (Luo, 2000). Over the past few years, Rho GTPases have been implicated in neuronal processes including neuronal migration and polarization, axon guidance, dendrite formation as well as synaptic organization and plasticity (comprehensively reviewed in (Luo, 2000). Given the large number of GEFs and GAPs shown to be functional and/or expressed in the nervous system, and others that are predicted on the basis of the available genome sequences, it is likely that Rho GTPase activity is intrinsic to various signaling pathways involved in the regulation of neuronal processes. Many of these pathways will be responsive to extracellular cues and stimuli to evoke cytoskeletal rearrangements, which underly detectable morphological adjustments in cells of the nervous system.

Through means of positional cloning about 10 genes have been identified thus far, offering the first mutational base to study the specific genetics and biochemistry involved in MRX. Among these, 3 MRX genes encode elements of potential Rho GTPase signaling pathways that are active in neurons. Others may be physiologically linked to Rho-function.

### *3.1. Oligophrenin-1*

The first Rho-related MRX gene to be identified was the oligophrenin-1 gene. It encodes a putative RhoGAP protein with a cannonical RhoGAP catalytic domain, which is likely to have a negative effect on Rho GTPase signaling. Two different mutations in the oligophrenin-1 gene have been isolated, all of which are supposed to lead to a loss-of-function phenotype (Antonarakis and Van Aelst, 1998; Billuart et al., 1998). So far, it remains incompletely understood as to which of the Rho-GTPases is the physiologically relevant target of oligophrenin-1 *in vivo* although the protein exerts GAP activity towards

Rac, Cdc42 and RhoA *in vitro*. This observation emphasizes a role for oligophrenin-1 as a negative regulator of Rho GTPase activity. Nevertheless, more specifically designed experiments will have to address the *in vivo* substrate preference, subcellular localization as well as the nature of the protein network it is part of to understand the disease causing role of the isolated mutations. It is conceivable that oligophrenin-1 also fulfills extra-neuronal functions since it is expressed in other tissues besides the brain, albeit at lower levels (Billuart et al., 1998). Loss of oligophrenin-1 function in humans does not give rise to any discernible phenotypes suggesting other than MRX-specific phenotypes in affected individuals could be covered by functional redundancy of Rho GAPs in other tissues.

### *3.2. PAK3*

As a second Rho-related MRX-gene PAK3 has been described and two different mutations in the PAK3 genes have been isolated in two MRX-pedigrees. As described earlier, PAK kinases have been defined as Rac1- and Cdc42-specific effector molecules that activte downstream pathways from the activated Rho GTPases (Manser et al., 1994). PAK3 is highly expressed in MRX-relevant brain regions, namely the cortex and hippocampus, suggesting that PAK3 kinase function is required either for the development of these regions, or in the inherent synaptic plasticity in the synaptic spines in differentiated regions with particular cognitive tasks (Allen et al., 1998). PAK kinases, according to a current model, are thought to be cytoplasmically dormant in their inactive state. Upon recruitment by an upstream factor, which could be activated Rac1 or Cdc42, the autoinhibitory interaction between the N-terminal regulatory domain and the C-terminal catalytic domain is disrupted. This switches PAK into an active conformation capable of auto- and substrate phosphorylation (Aghazadeh et al., 1998; Bagrodia and Cerione, 1999; Buchwald et al., 2001). A point mutation present in a multiplex pedigree abrogates the Ser/Thr-directed kinase function by inserting a premature stop-codon (Allen et al., 1998). In another MRX pedigree, the missense mutation R67C has been implicated as a cause for moderate to severe mental retardation. This amino acid substitution resides in the predicted RhoGTPase-interaction motif of PAK3 and hints to the relevance of the GTPase/PAK interaction (Bienvenu et al., 2000). The Drosophila homolog of PAK has been implicated in developmental aspects of the nervous system such as neurogenesis and axonal pathfinding (Melzig et al., 1998; Hing et al., 1999; Newsome et al., 2000). Imperfectly understood downstream signaling pathways relay PAK-activity to the actin cytoskeleton and possibly the nucleus. The identification of the PAK-relevant kinase targets in MRX will greatly contribute to our understanding of the physiological correlation between Rho GTPase activities and cognitive functions.

So what are the molecular components linking PAK to cytoskeletal alterations in neurons? Different proteins have been hypothesized to serve as substrates for PAK's kinase activity in general and particularly neuronal situations. PAK, as part of its activation mechanism phosphorylates itself (Manser et al., 1994). Furthermore, LIMK (or Lim-domain-containing protein kinase) has been identified by Edwards et al. as a potential downstream mediator of PAK (Edwards et al., 1999). Upon phosphorylation by PAK, LIM-kinase in turn phosphorylates, and thus de-activates, the actin depolymerizing factor Cofilin (Arber et al., 1998; Yang et al., 1998). This study suggests that PAK

connects Rac/Cdc42 activity with LIM-kinase in the regulation of actin cytoskeletal dynamics. Interestingly, hemizygosity of the LIM-kinase 1 encoding locus on chromosome 7q11 is correlated with the autosomal William's syndrome, a disorder comprised of a complex of phenotypes which affects about 1 in 20,000 births. Features of this condition include weakened visuospacial constructive cognition and varying severities of mental retardation (Frangiskakis et al., 1996). Another substrate of PAK is MLCK (Myosin light chain kinase), which upon phosphorylation at critical residues exerts a kinase-activity itself that is directed at the myosin regulatory light chain (MRLC) (Sanders et al., 1999). This event is thought to inhibit actin/myosin motor complexes. Along these lines, MRLC has also been proposed to be a direct PAK target (Sells et al., 1999). Most of these initial biochemical observations have come from the study of fibroblasts or epithelial cell lines. Thus, these different findings will have to be re-evaluated as to their importance in neurons in order to elucidate the relevant mechanisms involving the loss of PAK3 in MRX.

### *3.3. ARHGEF6*

Another intriguing addition to the list of MRX-causing mutations is the ARHGEF6 gene discovered by Kutsche and collegues (Kutsche et al., 2000). The ARHGEF6 gene product is identical to the previously discovered αPIX/Cool-2 protein was isolated as a PAK-binding partner that by means of biochemical co-purification and yeast-2-hybrid approaches. In fact, a small protein family containing αPIX/Cool-2 itself, βPIX/p85Cool-1 and its smaller splice variant p50Cool-1, has emerged (Bagrodia et al., 1998; Manser et al., 1998; Bagrodia et al., 1999). Interestingly, αPIX/Cool-2 also contains the typical tandem DH/PH motif that marks it as a potential RhoGEF. The potential for an interaction between αPIX/Cool-2 and PAK is mediated by an SH3 domain in the former and an unconventional SH3-binding site in the latter protein. αPIX/Cool-2, when co-expressed with PAK, can trigger PAK's kinase activity (Bagrodia et al., 1998; Daniels et al., 1999). Attempts to monitor directly the levels of [$^3$H] GDP released from Rac1 or Cdc42 did not doubtlessly reveal an exchange activity towards either of the GTPases. However, in *in vitro* assays αPIX/Cool-2 can increase the amount of GTP-bound Cdc42, whereas its sibling βPIX/p85Cool-1 can increase GTP-levels bound to Rac1. This activity, however, is significantly lower than that of the well-studied RhoGEF Dbl (Bagrodia et al., 1998; Manser et al., 1998; Bagrodia et al., 1999; Daniels et al., 1999). Taken together, the expression and biochemical experiments performed so far suggest that αPIX/Cool-2 can enhance PAK recruitment and activity but do not give an unambiguous answer as to the exact molecular mechanisms governing the GTPase/αPIX/Cool-2/PAK complex. Most probably, other factors and precise subcellular conditions dictate the function of this complex in various *in vivo* settings. As in the case of PAK3, the concrete cellular effects of the ARHGEF6 mutations requires further study.

### *3.4. IL1RAPL, TM4SF2 and FMRP*

Besides the above described genes that can be regarded as elements of Rho GTPase signaling pathways, there is a group of mental retardation genes that, although without

being evident Rho-signaling components, still may be indirectly linked to the function of Rho GTPases. IL1RAPL, TM4SF2 and FMRP code for a novel member of the interleukin-1 (IL-1) receptor family, a protein of the tetraspanin family of membrane proteins and an RNA-binding protein, respectively (Carrie et al., 1999; Zemni et al., 2000). It has been reported that IL-1, an inflammatory cytokine, can stimulate Cdc42 through its cognate IL-1 RI and RII receptors in fibroblasts. As a consequence, Rac and Rho GTPases also become activated. In fact, Cdc42 activity is absolutely required for IL-1-induced actin polymerisation and remodeling processes (Puls et al., 1999). Exposure of HeLa cells to IL-1 results in the activation of RhoA, which in turn leads to the formation of stress fibres. Moreover, IL-1 RI, in a biochemical affinity purification protocol, was found to be complexed with RhoA and Rac1 (Singh et al., 1999). It will be interesting to see, whether the newly identified MRX-related IL1RAP protein also will involve Rho GTPases as part of its general signaling potential in the brain.

Tetraspanins, on the other hand, have been observed in protein complexes with adhesion molecules such as α- and β-integrins. The TM4SF proteins CD151, CD81 and CD63 have been found to be associated with α3β1-integrins in neurites and growth cones of human NT2N cells. When treated with antibodies against CD151 and CD81, neurite outgrowth in NT2N cells grown on α3β1 integrin-specific ECM molecules was greatly impaired. Under these conditions, neurite number, length and extension rate where all affected (Stipp and Hemler, 2000). As there appears to be a general requirement for Rho GTPase activity in axon and neurite outgrowth and retraction, as well as a functional connection between integrin and Rho signaling it would not be surprising to find that TM4SF/integrin complexes function in concert with the Rho GTPases (Jalink et al., 1994; Luo et al., 1994). Intriguingly, in a recent study, a TM4SF-family member was netted as a binding partner of oligodendrocyte-specific protein (OSP/Claudin-11), a member of the expanding Claudin family. OSP/Claudin-11 is responsible for the establishing tight junction-equivalent structures in myelin- forming oligodendrocytes of the CNS. For this reason, the respective TM4SF protein was named OAS for OSP/Claudin-11 associated protein 1 (Tiwari-Woodruff et al., 2001). This complex was confirmed by co-immunoprecipitation and immunolocalization experiments. Furthermore, β1-integrin subunits were recovered as another constituent of OAS/OSP complexes (Tiwari-Woodruff et al., 2001). Loss of OSP/Claudin-11 in gene-targeted mice results in the absence of tight junction-like structures in CNS myelin sheaths, which induces neurological abnormalities (Gow et al., 1999). Interference experiments using OAP-1- and OSP/Claudin-11-specific antibodies in primary oligodendrocytes effectively impaired migration, whereas, overexpression of OAP-1 or OSP/Claudin-11 caused an oligodendrocytic cell line to overproliferate in culture (Tiwari-Woodruff et al., 2001). In light of the well-established role of Rho proteins in determining the morphology and function of tight junctions in epithelial cells (Jou et al., 1998), these data may hint at the possibility of oligodendrocytic defects being important in the genesis of MRX. It remains to be seen which specific cell type TMS4SF2 will be functional in. Its wide spread expression pattern so far does not exclude any of the mentioned possibilities, all of which may well be molecularly an physiologically linked to Rho GTPase signaling.

Finally, a potentially intriguing role for Rac1 signaling in the development of the fragile X mental retardation syndrom has been suggested (Schenck et al., 2001). FMRP (fragile X-linked mental retardation protein) is the product of the FMR1 gene, mutations

in which manifest themselves in the Fragile X Mental Retardation Syndrome (FraX). FraX can be distinguished from non-syndromic X-linked mental retardation in that it is also associated with other phenotypes such as macroorchidism, large ears, prominent jaws and a high-pitched jocular speech in affected individuals (Hagerman, 1996; Imbert *et al.*, 1998). In FraX, the FMR1 gene is transcriptionally silenced due to a hypermethylated CGG repeat expansion in the sequence encoding the 5'-untranslated region (Imbert *et al.*, 1998). FMRP harbors nuclear export and import signals, as well as multiple mRNA binding motifs (Ashley et al., 1993; Siomi et al., 1993; Eberhart et al., 1996; Sittler et al., 1996; Tamanini et al., 1999). Thus it may serve to shuttle specific mRNAs between the nucleus and the cytoplasm. The finding that the concentration of FMRP is high in neurons and particularly within dendritic processes suggests that it may direct its cargo mRNAs to a specialized cellular compartment for more efficient translation (Devys et al., 1993; Weiler et al., 1997; Jin and Warren, 2000). More recently, FMRP has been shown to associate with CYFIP1 (*c*ytoplasmic *F*MRP *i*nteracting *p*rotein 1), a component of the synaptosome (Schenck et al., 2001). In fact, CYFIP1 was a known protein that earlier had been described as a Rac1 interacting protein, termed p140Sra-1, which was observed to be associated with Rac-induced cortical actin filaments and to cosediment with F-actin (Kobayashi et al., 1998). It is tempting to speculate that activated Rac1, together with CYFIP1, tethers FMRP to developing spines to spacially control the translation of spine-relevant cargo mRNAs. Rac1 activation, in this scenario, may result from stimulation via activation of receptors for neurotransmitters in spine synapses. Mutational down-regulation of FMRP may interfere with such synthetic processes which ultimately produces FraX-associated phenotypes.

As illustrated above, a number of MRX genes are either evident components of Rho GTPase signaling cascades or may be indirectly linked to the activities of Rho GTPases. Since each of the cloned MRX genes accounts for only 0.5-1.0% of the total MRX-cases (Chelly, 2000), one can predict a considerable number of additional MRX genes to surface in the future. It is very likely that among these MRX genes additional components of Rho GTPase signaling pathways will be identified. Tying in MRX-defects on their physiological level with the molecular details of Rho GTPase signaling will be a major challenge. And moreover, finding out whether the Rho GTPase-related MRX genes and their products found thus far may cooperate in a common pathway is an interesting starting point. Also the development of animal models carrying targeted disruptions in MRX genes and transgenes that can be conditionally expressed will be beneficial to these aims. Defining these Rho GTPase-controlled pathways and integrating them into a general neuronal sigaling network will contribute to understanding the molecular details of cognitive processes.

## 4. Other potentially RhoGTPase related disabilities

### *4.1. FGD1 (Faciogenital Dysplasia)*

By conventional means of forward genetics the FGD1 gene has been cloned and revealed to be the locus involved in mutations causing the faciogenital dysplasia, also

known as Aarskog-Scott syndrome (Pasteris et al., 1994). The discovery of additional mutant FGD1-alleles since has confirmed the role of the gene in the development of the disease (Orrico et al., 2000; Schwartz et al., 2000). Faciogenital dysplasia is an X-linked developmental disorder and individuals are of disproportionately short stature and suffer from facial, skeletal and urogenital abnormalities. The FGD gene product, FGD1, was predicted to function as a Rho-specific GEF, and the disease-causing mutation at first identified by Pasteris *et al.* was predicted to insert a premature stop codon into the region encoding the protein's DH-function. FGD1, apart from the DH-motif also contains the juxtaposed PH-domain typical of Rho GEFs, as well as a number of SH3-binding regions and an additional C-terminal PH-domain (Pasteris et al., 1994). Subsequently, Zheng and collegues have demonstrated that FGD1 exerts its activity specifically on Cdc42 (Zheng et al., 1996). Further dissection of the FGD1 molecule determined that the DH-domain alone can induce G1 progression when introduced into fibroblasts, but that both DH and and the contingent PH sequence are needed to induce a Cdc42-specific response of the actin cytoskeleton, namely the formation of filopodia and microspikes (Nagata et al., 1998). This result suggests that proper recruitment of FGD1 to actin-relevant subcellular sites depends on proper PH-function. Interestingly, mutational analysis of another FGD1 pedigree by Orrico *et al.* has revealed a R610Q amino acid substitution (Orrico et al., 2000). Arg-610 resides in the PH-domain of the DH/PH-motif and appears to be a conserved residue in PH-domains that are involved in inositol-phosphate (InsP)-binding (Ferguson et al., 1995; Salim et al., 1996). Together, these results suggest that FGD1 requires to be targeted by specific InsP-species to distinct membranes where it exerts its GEF activity towards Cdc42. Up to date, no upstream activating mechanism for FGD1-specific signaling has been described but the strong similarity between the PH-domain harboring Arg-610 and the one of the β-adrenergic receptor kinase (ARK) indicates that PtdIns-4,5-$P_2$ and/or PtdIns-3,4,5-$P_3$ might be inductive agents. ARK has been shown to associate with these InsP species (Rameh et al., 1997).

*4.2. WASP (Wiskott-Aldrich Syndrome)*

Two other genetic disorders in which the responsible mutations affect Cdc42 signaling are the X-linked Wiskott-Aldrich syndrome (WAS) and the related allelic X-linked thrombocytopenia (XLT). As FGD and the MRX genes, the gene causing WAS and XLT (hence referred to as WAS) is also located on the X-chromosome, rendering recessive alleles (approximately 50 different mutations so far have been detected in the WAS gene) to phenotypic expression (Derry et al., 1995; Kolluri et al., 1995; Kwan et al., 1995; Villa et al., 1995; Wengler et al., 1995; Zhu et al., 1995). Defects are restricted to hematopoetic lineages and include microthrombocytopenia and recurrent infections because of dysregulated T- and B-cell functions. The observations that neutrophils are inabled in their proper chemotactic response and that B-cells do not show their typical response to polysaccarides together with the severe cytoskeletal defects observed in T-cells and platelets suggested an actin-related role for the WAS gene product. Derry et al. succeeded in cloning the gene responsible for WAS and through rigorous screening assembled the first set of WAS-specific mutations including nonsense, missense and frame shift mutations (Derry et al., 1994). Subsequently, in biochemical overlay assays designed to find novel Cdc42-effectors, Symons et al. isolated WASP (for WAS protein)

from human neutrophils (Symons et al., 1996). WASP appeared to bind exclusively to GTP-loaded Cdc42 and not to Rac1 or RhoA, and was traced in high concentrations to polymeric actin structures. Through the work of several groups a picture emerged in which Cdc42, in concert with WASP, stimulates the Arp2/3 complex which in turn enables actin filaments to nucleate from germination centers (for review see (Welch, 1999). This activity is required for morphological cell shape changes and cell migration, processes that are impaired in WAS-deficient hematopoetic cells. Accordingly, WASP transcripts were found in compartments of the immune systems, such as the spleen, thymus and lymphocytes (Derry et al., 1994). Notably, a homologue of WASP, namely N-WASP is expressed ubiquitously, with particularly high expression levels in the nervous system, and fulfills WASP functions in cells other than those of hematopoetic origin (Miki et al., 1996). In subsequent experiments, N-WASP was shown to be an indispensable element in the Cdc42-dependent pathway leading to the formation of filopodia in fibroblasts (Miki et al., 1998). WASP, moreover, appears to be subject to precise regulatory mechanisms to guarantee its proper function. This has become even more apparent when Devriendt et al. recently reported that another hematopoetic deficiency, namely X-linked severe congenital neutropenia (XLN) is caused by a WAS mutation that generates a constitutively activated WASP protein (Devriendt et al., 2001). A single amino acid substitution at position 270 from Leu to Pro in the autoinhibitory domain of WASP was detected and based on structural data by Kim and co-workers (Kim et al., 2000) is shown to map to the Rho GTPase binding domain (Kim et al., 2000; Devriendt et al., 2001). These findings underscore the *in vivo* importance of the Cdc42/WASP interaction once more.

*4.3. Diaphanous (non sydromic deafness)*

Another example of a gene encoding a disease-related Rho GTPase effector is Diaphanous 1 (also known as DFNA1). An effort of identifying the autosomal dominant mutation responsible for nonsyndromic deafness led to cloning of a gene on chromosome 5q31 that turned out to be homologous to the *Drosophila diaphanous* gene (Lynch et al., 1997). The first clue hinting at a possible function for diaphanous came from another direction. Watanabe and co-workers, in a quest for RhoA-specific binding partners, identified p140mDia/mDia1, the mouse orthologue of the diaphanous protein. They further showed that mDia1 was not only tethered to activated Rho by its N-terminus, but that it also serves as a ligand for Profilin via its formin homology (FH1) domain (Watanabe et al., 1997). This interaction is believed to stimulate actin remodeling at particular locations in the cell in response to Rho activation. Indeed, in a follow-up study mDia1 turned out to work in concert with ROCK to induce stress fibers in transfected fibroblasts (Watanabe et al., 1999). Interestingly, mDia1 caused the alignment of actin together with microtubule filaments in a coordinated bipolar fashion when transfected into HeLa cells. This activity was ascribed to the C-terminal FH2 domain of the molecule and suggests mDia1/diaphanous as an actin/microtubule coordinating factor downstream of Rho (Ishizaki et al., 2001). Deafness, arising from a mutations in the diaphanous gene is associated with a sensorineural cochleosaccular dysplasia of the membranous structures of the inner ear. It has been speculated that diaphanous may act in a pathway linking integrins to the actin cytoskeleton. Mutations in $\alpha 8\beta 1$ integrins,

diaphanous and myosinVIIa (as well as other myosins) all cause deafness indicating that the wild type proteins could organize and/or maintain cytoskeletal structures in hair cells of the inner ear (Lynch et al., 1997; Richardson et al., 1999; Littlewood Evans and Muller, 2000); for a review about deafness-related genes see (Muller and Littlewood-Evans, 2001).

*4.4. A potential role of Rho GTPases in Tangier disease*

In the last "case study" presented in this review, Rho GTPase function has been correlated with Tangier disease. The basis of this disease is an impairment in a cholesterol efflux (CE) mechanism resulting in abnormally low high density lipoprotein (HDL) plasma levels and an accumulation of cellular cholesteryl esters. Thus, CE is an essential process that purges excess cholesterol from cells and presents an important protection system against artheriosklerosis. Examination of fibroblasts and macrophages from Tangier patients by cDNA subtraction techniques revealed a substantial downregulation of Cdc42 levels in affected cells. Accordingly, Hirano et al. found that introduction of a dominant negative form of Cdc42 into epithelial MDCK cells could decrease CE efficiency and that dominant active Cdc42 could increase it (Hirano et al., 2000). In addition, RhoA, RhoB, RhoG and Rac1 levels appeared to be elevated in fibroblasts derived from Tangier patients (Utech et al., 2001). Whether Rho GTPases have a direct role in Tangier disease and how a particular Rho GTPase affects aspects of CE remains elusive, but future research into this subject may well shed light onto yet another aspect of Rho GTPase signaling.

*4.5. Rho GTPases and bacterial infections*

Through work in the last few years, it has become clear that the manipulation of Rho GTPases is a step in a number of disease-causing bacterial infections. Rho GTPases are manipulated by bacterial toxins of quite different origin, make and function. For more detail, we refer to recent reviews that treat this subject in much greater depth (Lerm et al., 2000; Stebbins and Galan, 2001). To summarize the recent research, Rho GTPases are targeted by basically three classes of toxins that display fundamentally different activities. The first class contains the Rho GTPase-inactivating C3 exotoxins and LCC toxins that act as ADP-ribosyltransferases and glucosyltransferases, respectively. The covalent attachment of ADP-ribosyl-moieties by the C3-transferases of *Clostridium botulinum*, *Staphylococcus aureus* and other infectious bacteria blocks the ability of RhoA, B and C to interact with Rho-specific GEFs and thus prevents activation of the GTPases. Glucosylation of Rho at Thr-37 and Rac and Cdc42 at Thr-35 by the large LCC toxins (larger than 250 kD) which is produced by *Clostridium spp.* interferes with nucleotide binding and coordination of the nucleotide-linked $Mg^{2+}$-ion. This, in turn inhibits the association of effector molecules with the same region of the GTPases and abrogates the stimulation of downstream signaling pathways. The second class of toxins contains the Rho GTPase-deamidating CNF agents from *E. coli* and DNT toxins from *Boretella spp.* The former ones deamidate Gln-63 in Rho and Gln-61 in Rac and Cdc42. Since these residues are essential for GTP-hydrolysis and therefore inactivation of the Rho GTPases, this renders them constitutively active. This results in an overactivation of

particular Rho-dependent signaling pathways. DNT toxins are transglutaminases that constitutively activate Rho by adding an amine to Gln-63. A particularly interesting class of toxins is the one of 'injected' toxins, that due to a specialized 'typeIII' secretion system are directly introduced from *Yersinia* and *Salmonella* bacteria into their host cells. 'Injected' toxins mimick cellular GEFs and GAPs to a degree that allows the invading bacterium to first activate Rho GTPases, such as Rac1 and Cdc42 in the case of SopE from Salmonella spp. This ensues remodeling of the actin cytoskeleton to promote bacterial entry. Post internalization, another class of 'injected' toxins, exemplified by SptP *from Salmonella spp.* and YopE from *Yersinia spp.* is produced, this time to reversibly inactivate Rho GTPases by mimicking host GAPs. Thus, deregulation of cytoskeletal structures is reversed and the host cell saved for the future benefit of the invadors. Recent structural analysis has unraveled the basis for some of these mechanisms and it appears, that through convergent evolution *Salmonella spp.* and *Yersinia spp.* have developed proteins that, although they do not share the tertiary structures of the enzymes they mimick, they nevertheless display contact surfaces and identical residues at critical positions that allow for catalysis (Lerm et al., 2000; Stebbins and Galan, 2001). The corruption of Rho GTPase activities in the interest of pathogenic infection can be regarded as a redundant theme and the different strategies that have been evolutionarily employed by bacteria to this end are diverse and fascinating. The observation that over- as well as underactivation of Rho GTPases occurs in the course of bacterial attacks suggests that Rho GTPase signaling is being exploited in some cases and disrupted in others. In this sense, the strategies used by *Salmonella spp.* seem even more sophisticated. The C3-transferase from *Clostridium botulinum* has been already used successfully as an experimental tool to inhibit Rho-function in diverse systems (see e.g. (Cleverley et al., 2000). It is conceivable, that other Rho GTPase-specific toxins will be introduced as more generally applicable reagents into research that pertains to clinically relevant questions.

## 5. Conclusions and future perspectives

Given the complexity of Rho GTPase signaling and the multiple cellular and developmental aspects involving and requiring the function of Rho GTPases, it is very strong a possibility that many more disease-causing mutations in genes encoding Rho-related signaling molecules will be uncovered in the future. The available annotated genome sequences suggest a vast number of genes for Rho GTPase-specific regulators but to date many of them remain biologically unexplored.

Insightful evidence is beginning to link deregulated Rho-signaling pathways to different cellular phenomena in the context of the clinical abnormalities diagnostic of the respective disorders. Further crosstalk and interdependencies between different Rho GTPase-regulated pathways themselves and other pathways are being discovered. The following points illustrate some of the possibilities that may allow further complexity and coherens. (1) The findings that Wnt- and Ras-signaling may feed into Rho-signaling, while using the latter for some of their manifestations are examples for interdependent signaling pathways. Other disease-relevant pathways are likely to be linked to Rho-signaling in the years to come. Adhesion-triggered signaling in various forms is a good candidate for this. (2) Rho-specific activities in the establishment of cellular polarity and

their impact on vesicle trafficking may be of general biological importance for different cellular systems. (3) Along the same lines, the MRX genes encoding components and potential components of Rho GTPase signaling pathways that have been identified thus far will have to be functionally connected to an operational network. The establishment of mouse model systems will help understanding the contribution of these genes and their interactions on the physiological level. It is evident that the biological activities of the disease genes elaborated upon in this review will exceed the scope of the context in which they were initially identified. Research, addressing these aspects may ultimately also help design strategies for therapeutic intervention and diagnostic purposes. Certainly, the interplay and collaboration between disease research on the one hand and biochemical as well as cellbiological research on the other will further help advance these subjects and activate our individual levels of excitement.

## Acknowledgements

We would like to Sarah Newey and Eve-Ellen Govek for their comments on the manuscript. Benjamin Boettner is a fellow of the "Gesellschaft der Naturforscher Leopoldina". Linda Van Aelst is supported by grants from the U.S. Army, the NIH and the NF Foundation Inc. Due to the complextity of the subject under review here, we would like to apologize to those collegues whose contributions have not been considerated.

## References

Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J. and Kumar, R.: Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. J Biol Chem 275 (2000) 12041-50.

Aghazadeh, B., Lowry, W.E., Huang, X.Y. and Rosen, M.K.: Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. Cell 102 (2000) 625-33.

Aghazadeh, B., Zhu, K., Kubiseski, T.J., Liu, G.A., Pawson, T., Zheng, Y. and Rosen, M.K.: Structure and mutagenesis of the Dbl homology domain. Nat Struct Biol 5 (1998) 1098-107.

Allen, K.M., Gleeson, J.G., Bagrodia, S., Partington, M.W., MacMillan, J.C., Cerione, R.A., Mulley, J.C. and Walsh, C.A.: PAK3 mutation in nonsyndromic X-linked mental retardation. Nat Genet 20 (1998) 25-30.

Antonarakis, S.E. and Van Aelst, L.: Mind the GAP, Rho, Rab and GDI. Nat Genet 19 (1998) 106-8.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P.: Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393 (1998) 805-9.

Ashley, C.T., Jr., Wilkinson, K.D., Reines, D. and Warren, S.T.: FMR1 protein: conserved RNP family domains and selective RNA binding. Science 262 (1993) 563-6.

Bagrodia, S., Bailey, D., Lenard, Z., Hart, M., Guan, J.L., Premont, R.T., Taylor, S.J. and Cerione, R.A.: A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. J Biol Chem 274 (1999) 22393-400.

Bagrodia, S. and Cerione, R.A.: Pak to the future. Trends Cell Biol 9 (1999) 350-5.

Bagrodia, S., Taylor, S.J., Jordon, K.A., Van Aelst, L. and Cerione, R.A.: A novel regulator of p21-activated kinases. J Biol Chem 273 (1998) 23633-6.

Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M. and Bartek, J.: Cyclin D1 protein expression and function in human breast cancer. Int J Cancer 57 (1994) 353-61.

Berra, E., Diaz-Meco, M.T., Dominguez, I., Municio, M.M., Sanz, L., Lozano, J., Chapkin, R.S. and Moscat, J.: Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell 74 (1993) 555-63.

Bienvenu, T., des Portes, V., McDonell, N., Carrie, A., Zemni, R., Couvert, P., Ropers, H.H., Moraine, C., van Bokhoven, H., Fryns, J.P., Allen, K., Walsh, C.A., Boue, J., Kahn, A., Chelly, J. and Beldjord, C.: Missense mutation in PAK3, R67C, causes X-linked nonspecific mental retardation. Am J Med Genet 93 (2000) 294-8.

Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M.C., Zemni, R., Roest Crollius, H., Carrie, A., Fauchereau, F., Cherry, M., Briault, S., Hamel, B., Fryns, J.P., Beldjord, C., Kahn, A., Moraine, C. and Chelly, J.: Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. Nature 392 (1998) 923-6.

Boquet, P.: Bacterial toxins inhibiting or activating small GTP-binding proteins. Ann N Y Acad Sci 886 (1999) 83-90.

Bos, J.L.: The ras gene family and human carcinogenesis. Mutat Res 195 (1988) 255-71.

Buchwald, G., Hostinova, E., Rudolph, M.G., Kraemer, A., Sickmann, A., Meyer, H.E., Scheffzek, K. and Wittinghofer, A.: Conformational switch and role of phosphorylation in PAK activation. Mol Cell Biol 21 (2001) 5179-89.

Bustelo, X.R.: Regulatory and signaling properties of the Vav family. Mol Cell Biol 20 (2000) 1461-77.

Carrie, A., Jun, L., Bienvenu, T., Vinet, M.C., McDonell, N., Couvert, P., Zemni, R., Cardona, A., Van Buggenhout, G., Frints, S., Hamel, B., Moraine, C., Ropers, H.H., Strom, T., Howell, G.R., Whittaker, A., Ross, M.T., Kahn, A., Fryns, J.P., Beldjord, C., Marynen, P. and Chelly, J.: A new member of the IL-1 receptor family highly expressed in hippocampus and involved in X-linked mental retardation. Nat Genet 23 (1999) 25-31.

Chelly, J.: MRX review. Am J Med Genet 94 (2000) 364-6.

Chen, F., Ma, L., Parrini, M.C., Mao, X., Lopez, M., Wu, C., Marks, P.W., Davidson, L., Kwiatkowski, D.J., Kirchhausen, T., Orkin, S.H., Rosen, F.S., Mayer, B.J., Kirschner, M.W. and Alt, F.W.: Cdc42 is required for PIP(2)-induced actin polymerization and early development but not for cell viability. Curr Biol 10 (2000) 758-65.

Cheng, M., Sexl, V., Sherr, C.J. and Roussel, M.F.: Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc Natl Acad Sci U S A 95 (1998) 1091-6.

Chiariello, M., Marinissen, M.J. and Gutkind, J.S.: Regulation of c-myc expression by PDGF through Rho GTPases. Nat Cell Biol 3 (2001) 580-6.

Clark, E.A., Golub, T.R., Lander, E.S. and Hynes, R.O.: Genomic analysis of metastasis reveals an essential role for RhoC. Nature 406 (2000) 532-5.

Cleverley, S.C., Costello, P.S., Henning, S.W. and Cantrell, D.A.: Loss of Rho function in the thymus is accompanied by the development of thymic lymphoma. Oncogene 19 (2000) 13-20.

Coniglio, S.J., Jou, T.S. and Symons, M.: Rac1 protects epithelial cells against anoikis. J Biol Chem 276 (2001) 28113-20.

Costello, P.S., Cleverley, S.C., Galandrini, R., Henning, S.W. and Cantrell, D.A.: The GTPase rho controls a p53-dependent survival checkpoint during thymopoiesis. J Exp Med 192 (2000) 77-85.

Daniels, R.H., Zenke, F.T. and Bokoch, G.M.: alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. J Biol Chem 274 (1999) 6047-50.

Derry, J.M., Kerns, J.A., Weinberg, K.I., Ochs, H.D., Volpini, V., Estivill, X., Walker, A.P. and Francke, U.: WASP gene mutations in Wiskott-Aldrich syndrome and X-linked thrombocytopenia. Hum Mol Genet 4 (1995) 1127-35.

Derry, J.M., Ochs, H.D. and Francke, U.: Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell 78 (1994) 635-44.

Devriendt, K., Kim, A.S., Mathijs, G., Frints, S.G., Schwartz, M., Van Den Oord, J.J., Verhoef, G.E., Boogaerts, M.A., Fryns, J.P., You, D., Rosen, M.K. and Vandenberghe, P.: Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. Nat Genet 27 (2001) 313-7.

Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P. and Mandel, J.L.: The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4 (1993) 335-40.

Diaz-Meco, M.T., Municio, M.M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J.: The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. Cell 86 (1996) 777-86.

Dickson, C., Fantl, V., Gillett, C., Brookes, S., Bartek, J., Smith, R., Fisher, C., Barnes, D. and Peters, G.: Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. Cancer Lett 90 (1995) 43-50.

Dominguez, I., Sanz, L., Arenzana-Seisdedos, F., Diaz-Meco, M.T., Virelizier, J.L. and Moscat, J.: Inhibition of protein kinase C zeta subspecies blocks the activation of an NF-kappa B-like activity in Xenopus laevis oocytes. Mol Cell Biol 13 (1993) 1290-5.

Eberhart, D.E., Malter, H.E., Feng, Y. and Warren, S.T.: The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. Hum Mol Genet 5 (1996) 1083-91.

Edwards, D.C., Sanders, L.C., Bokoch, G.M. and Gill, G.N.: Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol 1 (1999) 253-9.

Engers, R., Zwaka, T.P., Gohr, L., Weber, A., Gerharz, C.D. and Gabbert, H.E.: Tiam1 mutations in human renal-cell carcinomas. Int J Cancer 88 (2000) 369-76.

Erickson, J.W., Zhang, C., Kahn, R.A., Evans, T. and Cerione, R.A.: Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J Biol Chem 271 (1996) 26850-4.

Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B.: Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. Cell 83 (1995) 1037-46.

Finco, T.S., Westwick, J.K., Norris, J.L., Beg, A.A., Der, C.J. and Baldwin, A.S., Jr.: Oncogenic Ha-Ras-induced signaling activates NF-kappaB transcriptional activity, which is required for cellular transformation. J Biol Chem 272 (1997) 24113-6.

Frangiskakis, J.M., Ewart, A.K., Morris, C.A., Mervis, C.B., Bertrand, J., Robinson, B.F., Klein, B.P., Ensing, G.J., Everett, L.A., Green, E.D., Proschel, C., Gutowski, N.J., Noble, M., Atkinson, D.L., Odelberg, S.J. and Keating, M.T.: LIM-kinase1 hemizygosity implicated in impaired visuospatial constructive cognition. Cell 86 (1996) 59-69.

Fritz, G., Just, I. and Kaina, B.: Rho GTPases are over-expressed in human tumors. Int J Cancer 81 (1999) 682-7.

Fukuhara, S., Chikumi, H. and Gutkind, J.S.: Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. FEBS Lett 485 (2000) 183-8.

Gille, H. and Downward, J.: Multiple ras effector pathways contribute to G(1) cell cycle progression. J Biol Chem 274 (1999) 22033-40.

Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., Barnes, D. and Peters, G.: Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 54 (1994) 1812-7.

Gow, A., Southwood, C.M., Li, J.S., Pariali, M., Riordan, G.P., Brodie, S.E., Danias, J., Bronstein, J.M., Kachar, B. and Lazzarini, R.A.: CNS myelin and sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. Cell 99 (1999) 649-59.

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A. and Collard, J.G.: Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell 77 (1994) 537-49.

Hagerman, R.J.: in Fragile X Syndrome: Diagnosis, Treatment and Research, eds. Hagerman, R.J. and Cronister-Silverman, A., John Hopkins Univ. Press, Baltimore. (1996) 3-68.

Hall, A.: Rho GTPases and the actin cytoskeleton. Science 279 (1998) 509-14.

Hing, H., Xiao, J., Harden, N., Lim, L. and Zipursky, S.L.: Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. Cell 97 (1999) 853-63.

Hinton, V.J., Brown, W.T., Wisniewski, K. and Rudelli, R.D.: Analysis of neocortex in three males with the fragile X syndrome. Am J Med Genet 41 (1991) 289-94.

Hirano, K., Matsuura, F., Tsukamoto, K., Zhang, Z., Matsuyama, A., Takaishi, K., Komuro, R., Suehiro, T., Yamashita, S., Takai, Y. and Matsuzawa, Y.: Decreased expression of a member of the Rho GTPase family, Cdc42Hs, in cells from Tangier disease - the small G protein may play a role in cholesterol efflux. FEBS Lett 484 (2000) 275-9.

Hordijk, P.L., ten Klooster, J.P., van der Kammen, R.A., Michiels, F., Oomen, L.C. and Collard, J.G.: Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. Science 278 (1997) 1464-6.

Hu, W., Bellone, C.J. and Baldassare, J.J.: RhoA stimulates p27(Kip) degradation through its regulation of cyclin E/CDK2 activity. J Biol Chem 274 (1999) 3396-401.

Imbert, G., Feng, Y., Nelson, D., Warren, S.T. and Mandel, J.-L.: in Genetic Instabilities and Hereditary Neurological Diseases, eds. Warren, S.T. and Wells, R.D., Academic, San Diego. (1998) 27-53.

Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T. and Narumiya, S.: Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. Nat Cell Biol 3 (2001) 8-14.

Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T. and Narumiya, S.: An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 5 (1999) 221-5.

Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H.: Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126 (1994) 801-10.

Jin, P. and Warren, S.T.: Understanding the molecular basis of fragile X syndrome. Hum Mol Genet 9 (2000) 901-8.

Joneson, T. and Bar-Sagi, D.: Suppression of Ras-induced apoptosis by the Rac GTPase. Mol Cell Biol 19 (1999) 5892-901.

Joneson, T., McDonough, M., Bar-Sagi, D. and Van Aelst, L.: RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science 274 (1996) 1374-6.

Jou, T.S., Schneeberger, E.E. and Nelson, W.J.: Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. J Cell Biol 142 (1998) 101-15.

Joyce, D., Bouzahzah, B., Fu, M., Albanese, C., D'Amico, M., Steer, J., Klein, J.U., Lee, R.J., Segall, J.E., Westwick, J.K., Der, C.J. and Pestell, R.G.: Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. J Biol Chem 274 (1999) 25245-9.

Kamai, T., Arai, K., Tsujii, T., Honda, M. and Yoshida, K.: Overexpression of RhoA mRNA is associated with advanced stage in testicular germ cell tumour. BJU Int 87 (2001) 227-31.

Kantor, J.D., McCormick, B., Steeg, P.S. and Zetter, B.R.: Inhibition of cell motility after nm23 transfection of human and murine tumor cells. Cancer Res 53 (1993) 1971-3.

Kerkhoff, E. and Rapp, U.R.: Cell cycle targets of Ras/Raf signalling. Oncogene 17 (1998) 1457-62.

Khosravi-Far, R., Solski, P.A., Clark, G.J., Kinch, M.S. and Der, C.J.: Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol 15 (1995) 6443-53.

Kim, A.S., Kakalis, L.T., Abdul-Manan, N., Liu, G.A. and Rosen, M.K.: Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. Nature 404 (2000) 151-8.

Kobayashi, K., Kuroda, S., Fukata, M., Nakamura, T., Nagase, T., Nomura, N., Matsuura, Y., Yoshida-Kubomura, N., Iwamatsu, A. and Kaibuchi, K.: p140Sra-1

(specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. J Biol Chem 273 (1998) 291-5.

Kolluri, R., Shehabeldin, A., Peacocke, M., Lamhonwah, A.M., Teichert-Kuliszewska, K., Weissman, S.M. and Siminovitch, K.A.: Identification of WASP mutations in patients with Wiskott-Aldrich syndrome and isolated thrombocytopenia reveals allelic heterogeneity at the WAS locus. Hum Mol Genet 4 (1995) 1119-26.

Kourlas, P.J., Strout, M.P., Becknell, B., Veronese, M.L., Croce, C.M., Theil, K.S., Krahe, R., Ruutu, T., Knuutila, S., Bloomfield, C.D. and Caligiuri, M.A.: Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. Proc Natl Acad Sci U S A 97 (2000) 2145-50.

Kroschewski, R., Hall, A. and Mellman, I.: Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat Cell Biol 1 (1999) 8-13.

Kutsche, K., Yntema, H., Brandt, A., Jantke, I., Nothwang, H.G., Orth, U., Boavida, M.G., David, D., Chelly, J., Fryns, J.P., Moraine, C., Ropers, H.H., Hamel, B.C., van Bokhoven, H. and Gal, A.: Mutations in ARHGEF6, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation. Nat Genet 26 (2000) 247-50.

Kwan, S.P., Hagemann, T.L., Radtke, B.E., Blaese, R.M. and Rosen, F.S.: Identification of mutations in the Wiskott-Aldrich syndrome gene and characterization of a polymorphic dinucleotide repeat at DXS6940, adjacent to the disease gene. Proc Natl Acad Sci U S A 92 (1995) 4706-10.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A.: Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell 87 (1996) 519-29.

Lamb, R.F., Roy, C., Diefenbach, T.J., Vinters, H.V., Johnson, M.W., Jay, D.G. and Hall, A.: The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. Nat Cell Biol 2 (2000) 281-7.

Leone, A., McBride, O.W., Weston, A., Wang, M.G., Anglard, P., Cropp, C.S., Goepel, J.R., Lidereau, R., Callahan, R., Linehan, W.M. and et al.: Somatic allelic deletion of nm23 in human cancer. Cancer Res 51 (1991) 2490-3.

Lerm, M., Schmidt, G. and Aktories, K.: Bacterial protein toxins targeting rho GTPases. FEMS Microbiol Lett 188 (2000) 1-6.

Lin, R., Bagrodia, S., Cerione, R. and Manor, D.: A novel Cdc42Hs mutant induces cellular transformation. Curr Biol 7 (1997) 794-7.

Littlewood Evans, A. and Muller, U.: Stereocilia defects in the sensory hair cells of the inner ear in mice deficient in integrin alpha8beta1. Nat Genet 24 (2000) 424-8.

Liu, A., Cerniglia, G.J., Bernhard, E.J. and Prendergast, G.C.: RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage. Proc Natl Acad Sci U S A 98 (2001) 6192-7.

Luo, L.: Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci 1 (2000) 173-80.

Luo, L., Liao, Y.J., Jan, L.Y. and Jan, Y.N.: Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 8 (1994) 1787-802.

Lynch, E.D., Lee, M.K., Morrow, J.E., Welcsh, P.L., Leon, P.E. and King, M.C.: Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the Drosophila gene diaphanous. Science 278 (1997) 1315-8.

MacDonald, N.J., Freije, J.M., Stracke, M.L., Manrow, R.E. and Steeg, P.S.: Site-directed mutagenesis of nm23-H1. Mutation of proline 96 or serine 120 abrogates its motility inhibitory activity upon transfection into human breast carcinoma cells. J Biol Chem 271 (1996) 25107-16.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S. and Lim, L.: A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature 367 (1994) 40-6.

Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T. and Lim, L.: PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol Cell 1 (1998) 183-92.

Matus, A.: Postsynaptic actin and neuronal plasticity. Curr Opin Neurobiol 9 (1999) 561-5.

Mayo, M.W., Wang, C.Y., Cogswell, P.C., Rogers-Graham, K.S., Lowe, S.W., Der, C.J. and Baldwin, A.S., Jr.: Requirement of NF-kappaB activation to suppress p53-independent apoptosis induced by oncogenic Ras. Science 278 (1997) 1812-5.

McIntosh, G.G., Anderson, J.J., Milton, I., Steward, M., Parr, A.H., Thomas, M.D., Henry, J.A., Angus, B., Lennard, T.W. and Horne, C.H.: Determination of the prognostic value of cyclin D1 overexpression in breast cancer. Oncogene 11 (1995) 885-91.

Melzig, J., Rein, K.H., Schafer, U., Pfister, H., Jackle, H., Heisenberg, M. and Raabe, T.: A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the Drosophila adult central nervous system. Curr Biol 8 (1998) 1223-6.

Mettouchi, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J.K. and Giancotti, F.G.: Integrin-Specific Activation of Rac Controls Progression through the G(1) Phase of the Cell Cycle. Mol Cell 8 (2001) 115-27.

Michiels, F., Habets, G.G., Stam, J.C., van der Kammen, R.A. and Collard, J.G.: A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature 375 (1995) 338-40.

Miki, H., Miura, K. and Takenawa, T.: N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. Embo J 15 (1996) 5326-35.

Miki, H., Sasaki, T., Takai, Y. and Takenawa, T.: Induction of filopodium formation by a WASP-related actin- depolymerizing protein N-WASP. Nature 391 (1998) 93-6.

Mira, J.P., Benard, V., Groffen, J., Sanders, L.C. and Knaus, U.G.: Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc Natl Acad Sci U S A 97 (2000) 185-9.

Muller, U. and Littlewood-Evans, A.: Mechanisms that regulate mechanosensory hair cell differentiation. Trends Cell Biol 11 (2001) 334-42.

Musch, A., Cohen, D., Kreitzer, G. and Rodriguez-Boulan, E.: cdc42 regulates the exit of apical and basolateral proteins from the trans-Golgi network. Embo J 20 (2001) 2171-9.

Nagata, K., Driessens, M., Lamarche, N., Gorski, J.L. and Hall, A.: Activation of G1 progression, JNK mitogen-activated protein kinase, and actin filament assembly by the exchange factor FGD1. J Biol Chem 273 (1998) 15453-7.

Newsome, T.P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., Debant, A. and Dickson, B.J.: Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in Drosophila. Cell 101 (2000) 283-94.

Olson, M.F., Paterson, H.F. and Marshall, C.J.: Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature 394 (1998) 295-9.

Orrico, A., Galli, L., Falciani, M., Bracci, M., Cavaliere, M.L., Rinaldi, M.M., Musacchio, A. and Sorrentino, V.: A mutation in the pleckstrin homology (PH) domain of the FGD1 gene in an Italian family with faciogenital dysplasia (Aarskog-Scott syndrome). FEBS Lett 478 (2000) 216-20.

Otsuki, Y., Tanaka, M., Yoshii, S., Kawazoe, N., Nakaya, K. and Sugimura, H.: Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. Proc Natl Acad Sci U S A 98 (2001) 4385-90.

Pasteris, N.G., Cadle, A., Logie, L.J., Porteous, M.E., Schwartz, C.E., Stevenson, R.E., Glover, T.W., Wilroy, R.S. and Gorski, J.L.: Isolation and characterization of the faciogenital dysplasia (Aarskog- Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. Cell 79 (1994) 669-78.

Peifer, M. and Polakis, P.: Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. Science 287 (2000) 1606-9.

Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R. and Lacal, J.C.: Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. Genes Dev 11 (1997) 463-75.

Polakis, P.: Wnt signaling and cancer. Genes Dev 14 (2000) 1837-51.

Preudhomme, C., Roumier, C., Hildebrand, M.P., Dallery-Prudhomme, E., Lantoine, D., Lai, J.L., Daudignon, A., Adenis, C., Bauters, F., Fenaux, P., Kerckaert, J.P. and Galiegue-Zouitina, S.: Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP- binding protein, in non-Hodgkin's lymphoma and multiple myeloma. Oncogene 19 (2000) 2023-32.

Price, L.S. and Collard, J.G.: Regulation of the cytoskeleton by Rho-family GTPases: implications for tumour cell invasion. Semin Cancer Biol 11 (2001) 167-73.

Pruitt, K. and Der, C.J.: Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett 171 (2001) 1-10.

Puls, A., Eliopoulos, A.G., Nobes, C.D., Bridges, T., Young, L.S. and Hall, A.: Activation of the small GTPase Cdc42 by the inflammatory cytokines TNF(alpha) and IL-1, and by the Epstein-Barr virus transforming protein LMP1. J Cell Sci 112 (1999) 2983-92.

Qiu, R.G., Abo, A., McCormick, F. and Symons, M.: Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol Cell Biol 17 (1997) 3449-58.

Qiu, R.G., Chen, J., Kirn, D., McCormick, F. and Symons, M.: An essential role for Rac in Ras transformation. Nature 374 (1995a) 457-9.

Qiu, R.G., Chen, J., McCormick, F. and Symons, M.: A role for Rho in Ras transformation. Proc Natl Acad Sci U S A 92 (1995b) 11781-5.

Qiu, R.G., Abo, A. and Steven Martin, G.: A human homolog of the C. elegans polarity determinant Par-6 links Rac and Cdc42 to PKCzeta signaling and cell transformation. Curr Biol 10 (2000) 697-707.

Rameh, L.E., Arvidsson, A., Carraway, K.L., 3rd, Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D.S., Chen, C.S. and Cantley, L.C.: A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J Biol Chem 272 (1997) 22059-66.

Reiss, A.L., Aylward, E., Freund, L.S., Joshi, P.K. and Bryan, R.N.: Neuroanatomy of fragile X syndrome: the posterior fossa. Ann Neurol 29 (1991) 26-32.

Reiss, A.L., Lee, J. and Freund, L.: Neuroanatomy of fragile X syndrome: the temporal lobe. Neurology 44 (1994) 1317-24.

Reuther, G.W., Lambert, Q.T., Booden, M.A., Wennerberg, K., Becknell, B., Marcucci, G., Sondek, J., Caligiuri, M.A. and Der, C.J.: Leukemia-associated Rho guanine nucleotide exchange factor, a Dbl family protein found mutated in leukemia, causes transformation by activation of RhoA. J Biol Chem 276 (2001) 27145-51.

Richardson, G.P., Forge, A., Kros, C.J., Marcotti, W., Becker, D., Williams, D.S., Thorpe, J., Fleming, J., Brown, S.D. and Steel, K.P.: A missense mutation in myosin VIIA prevents aminoglycoside accumulation in early postnatal cochlear hair cells. Ann N Y Acad Sci 884 (1999) 110-24.

Ridley, A.J.: Rho proteins: linking signaling with membrane trafficking. Traffic 2 (2001) 303-10.

Rudelli, R.D., Brown, W.T., Wisniewski, K., Jenkins, E.C., Laure-Kamionowska, M., Connell, F. and Wisniewski, H.M.: Adult fragile X syndrome. Clinico-neuropathologic findings. Acta Neuropathol 67 (1985) 289-95.

Ruggieri, R., Chuang, Y.Y. and Symons, M.: The small GTPase Rac suppresses apoptosis caused by serum deprivation in fibroblasts. Mol Med 7 (2001) 293-300.

Sahai, E., Ishizaki, T., Narumiya, S. and Treisman, R.: Transformation mediated by RhoA requires activity of ROCK kinases. Curr Biol 9 (1999) 136-45.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I., Driscoll, P.C., Waterfield, M.D. and Panayotou, G.: Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. Embo J 15 (1996) 6241-50.

Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F. and Collard, J.G.: Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J Cell Biol 143 (1998) 1385-98.

Sanders, L.C., Matsumura, F., Bokoch, G.M. and de Lanerolle, P.: Inhibition of myosin light chain kinase by p21-activated kinase. Science 283 (1999) 2083-5.

Schenck, A., Bardoni, B., Moro, A., Bagni, C. and Mandel, J.L.: A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. Proc Natl Acad Sci U S A 98 (2001) 8844-9.

Schmitz, A.A., Govek, E.E., Bottner, B. and Van Aelst, L.: Rho GTPases: signaling, migration, and invasion. Exp Cell Res 261 (2000) 1-12.

Schnelzer, A., Prechtel, D., Knaus, U., Dehne, K., Gerhard, M., Graeff, H., Harbeck, N., Schmitt, M. and Lengyel, E.: Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 19 (2000) 3013-20.

Schwartz, C.E., Gillessen-Kaesbach, G., May, M., Cappa, M., Gorski, J., Steindl, K. and Neri, G.: Two novel mutations confirm FGD1 is responsible for the Aarskog syndrome. Eur J Hum Genet 8 (2000) 869-74.

Sells, M.A., Boyd, J.T. and Chernoff, J.: p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. J Cell Biol 145 (1999) 837-49.

Sherr, C.J. and Roberts, J.M.: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13 (1999) 1501-12.

Singh, R., Wang, B., Shirvaikar, A., Khan, S., Kamat, S., Schelling, J.R., Konieczkowski, M. and Sedor, J.R.: The IL-1 receptor and Rho directly associate to drive cell activation in inflammation. J Clin Invest 103 (1999) 1561-70.

Siomi, H., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G.: The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell 74 (1993) 291-8.

Sittler, A., Devys, D., Weber, C. and Mandel, J.L.: Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of fmr1 protein isoforms. Hum Mol Genet 5 (1996) 95-102.

Somlyo, A.V., Bradshaw, D., Ramos, S., Murphy, C., Myers, C.E. and Somlyo, A.P.: Rho-kinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells. Biochem Biophys Res Commun 269 (2000) 652-9.

Srivastava, S.K., Wheelock, R.H., Aaronson, S.A. and Eva, A.: Identification of the protein encoded by the human diffuse B-cell lymphoma (dbl) oncogene. Proc Natl Acad Sci U S A 83 (1986) 8868-72.

Stebbins, C.E. and Galan, J.E.: Structural mimicry in bacterial virulence. Nature 412 (2001) 701-5.

Stipp, C.S. and Hemler, M.E.: Transmembrane-4-superfamily proteins CD151 and CD81 associate with alpha 3 beta 1 integrin, and selectively contribute to alpha 3 beta 1-dependent neurite outgrowth. J Cell Sci 113 (2000) 1871-82.

Sugihara, K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A. and Katsuki, M.: Rac1 is required for the formation of three germ layers during gastrulation. Oncogene 17 (1998) 3427-33.

Sulciner, D.J., Irani, K., Yu, Z.X., Ferrans, V.J., Goldschmidt-Clermont, P. and Finkel, T.: rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol Cell Biol 16 (1996) 7115-21.

Suwa, H., Ohshio, G., Imamura, T., Watanabe, G., Arii, S., Imamura, M., Narumiya, S., Hiai, H. and Fukumoto, M.: Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 77 (1998) 147-52.

Symons, M.: Rho family GTPases: the cytoskeleton and beyond. Trends Biochem Sci 21 (1996) 178-81.

Symons, M., Derry, J.M., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U. and Abo, A.: Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 84 (1996) 723-34.

Symons, M. and Settleman, J.: Rho family GTPases: more than simple switches. Trends Cell Biol 10 (2000) 415-9.

Takahashi, K., Sasaki, T., Mammoto, A., Hotta, I., Takaishi, K., Imamura, H., Nakano, K., Kodama, A. and Takai, Y.: Interaction of radixin with Rho small G protein GDP/GTP exchange protein Dbl. Oncogene 16 (1998) 3279-84.

Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S. and Takai, Y.: Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. J Biol Chem 272 (1997) 23371-5.

Tamanini, F., Bontekoe, C., Bakker, C.E., van Unen, L., Anar, B., Willemsen, R., Yoshida, M., Galjaard, H., Oostra, B.A. and Hoogeveen, A.T.: Different targets for the fragile X-related proteins revealed by their distinct nuclear localizations. Hum Mol Genet 8 (1999) 863-9.

Tang, Y., Chen, Z., Ambrose, D., Liu, J., Gibbs, J.B., Chernoff, J. and Field, J.: Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. Mol Cell Biol 17 (1997) 4454-64.

Tao, W., Pennica, D., Xu, L., Kalejta, R.F. and Levine, A.J.: Wrch-1, a novel member of the Rho gene family that is regulated by Wnt- 1. Genes Dev 15 (2001) 1796-807.

Tiwari-Woodruff, S.K., Buznikov, A.G., Vu, T.Q., Micevych, P.E., Chen, K., Kornblum, H.I. and Bronstein, J.M.: OSP/claudin-11 forms a complex with a novel member of the tetraspanin super family and beta1 integrin and regulates proliferation and migration of oligodendrocytes. J Cell Biol 153 (2001) 295-305.

Tran Quang, C., Gautreau, A., Arpin, M. and Treisman, R.: Ezrin function is required for ROCK-mediated fibroblast transformation by the Net and Dbl oncogenes. Embo J 19 (2000) 4565-76.

Utech, M., Hobbel, G., Rust, S., Reinecke, H., Assmann, G. and Walter, M.: Accumulation of RhoA, RhoB, RhoG, and Rac1 in fibroblasts from Tangier disease subjects suggests a regulatory role of Rho family proteins in cholesterol efflux. Biochem Biophys Res Commun 280 (2001) 229-36.

Van Aelst, L. and D'Souza-Schorey, C.: Rho GTPases and signaling networks. Genes Dev 11 (1997) 2295-322.

van Golen, K.L., Wu, Z.F., Qiao, X.T., Bao, L.W. and Merajver, S.D.: RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 60 (2000) 5832-8.

Villa, A., Notarangelo, L., Macchi, P., Mantuano, E., Cavagni, G., Brugnoni, D., Strina, D., Patrosso, M.C., Ramenghi, U., Sacco, M.G. and et al.: X-linked thrombocytopenia and Wiskott-Aldrich syndrome are allelic diseases with mutations in the WASP gene. Nat Genet 9 (1995) 414-7.

Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S.: Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat Cell Biol 1 (1999) 136-43.

Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B.M. and Narumiya, S.: p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. Embo J 16 (1997) 3044-56.

Weber, J.D., Hu, W., Jefcoat, S.C., Jr., Raben, D.M. and Baldassare, J.J.: Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27. J Biol Chem 272 (1997) 32966-71.

Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J. and Greenough, W.T.: Fragile X mental

retardation protein is translated near synapses in response to neurotransmitter activation. Proc Natl Acad Sci U S A 94 (1997) 5395-400.

Welch, M.D.: The world according to Arp: regulation of actin nucleation by the Arp2/3 complex. Trends Cell Biol 9 (1999) 423-7.

Wengler, G.S., Notarangelo, L.D., Berardelli, S., Pollonni, G., Mella, P., Fasth, A., Ugazio, A.G. and Parolini, O.: High prevalence of nonsense, frame shift, and splice-site mutations in 16 patients with full-blown Wiskott-Aldrich syndrome. Blood 86 (1995) 3648-54.

Westwick, J.K., Lambert, Q.T., Clark, G.J., Symons, M., Van Aelst, L., Pestell, R.G. and Der, C.J.: Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol Cell Biol 17 (1997) 1324-35.

Wu, W.J., Erickson, J.W., Lin, R. and Cerione, R.A.: The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature 405 (2000) 800-4.

Yang, F.C., Kapur, R., King, A.J., Tao, W., Kim, C., Borneo, J., Breese, R., Marshall, M., Dinauer, M.C. and Williams, D.A.: Rac2 stimulates Akt activation affecting BAD/Bcl-XL expression while mediating survival and actin function in primary mast cells. Immunity 12 (2000) 557-68.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K.: Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393 (1998) 809-12.

Yu, Q., Geng, Y. and Sicinski, P.: Specific protection against breast cancers by cyclin D1 ablation. Nature 411 (2001) 1017-21.

Zemni, R., Bienvenu, T., Vinet, M.C., Sefiani, A., Carrie, A., Billuart, P., McDonell, N., Couvert, P., Francis, F., Chafey, P., Fauchereau, F., Friocourt, G., Portes, V., Cardona, A., Frints, S., Meindl, A., Brandau, O., Ronce, N., Moraine, C., Bokhoven, H., Ropers, H.H., Sudbrak, R., Kahn, A., Fryns, J.P., Beldjord, C. and et al.: A new gene involved in X-linked mental retardation identified by analysis of an X;2 balanced translocation. Nat Genet 24 (2000) 167-70.

Zheng, Y., Fischer, D.J., Santos, M.F., Tigyi, G., Pasteris, N.G., Gorski, J.L. and Xu, Y.: The faciogenital dysplasia gene product FGD1 functions as a Cdc42Hs- specific guanine-nucleotide exchange factor. J Biol Chem 271 (1996) 33169-72.

Zhu, Q., Zhang, M., Blaese, R.M., Derry, J.M., Junker, A., Francke, U., Chen, S.H. and Ochs, H.D.: The Wiskott-Aldrich syndrome and X-linked congenital thrombocytopenia are caused by mutations of the same gene. Blood 86 (1995) 3797-804.

# [11] Ras and Rap1 Interaction with AF-6 Effector Target

*By* BENJAMIN BOETTNER, CHRISTIAN HERRMANN, and LINDA VAN AELST

## Introduction

The Rap types of small GTPases are members of the Ras superfamily and are the molecules that show the most identity with the oncogenic Ras proteins. Whereas the interaction of activated Ras proteins with their downstream effectors Raf, Ral guanine nucleotide dissociation stimulator (RalGDS), and phosphatidylinositol 3-kinase (PI3K) led to a fairly defined



METHODS IN ENZYMOLOGY, VOL. 332
0076-6879/01 $35.00

*Coimmunoprecipitation of RIN1 and RAS from Mammalian Cells*

In a previous study, we employed coimmunoprecipitation to detect RIN1–RAS complexes in mammalian cells that express both constitutively active RAS and RIN1 proteins.[6] We showed that RIN1 specifically binds to activated mammalian RAS in NIH 3T3 cells. Notably, the *in vivo* association of RIN1 with RAS was not detectable when using a RAS antibody that is directed at a portion of the effector domain. This result, similar to findings for RAF,[27] is consistent with a free effector domain requirement for RIN1 binding.

## Conclusions

Both genetic and biochemical approaches can be used to analyze the physical interaction of RIN1 and RAS. Results from various methodologies indicate that RIN1 binds to the effector domain and preferentially recognizes the activated conformation of RAS. The effector mutant binding profile of RIN1, together with its unique tissue expression pattern, distinguishes it from other known RAS effectors. Although no catalytic activity has been ascribed to RIN1, the presence of both ABL- and RAS-binding domains suggests a role in coordinating the transmission of signals through these pathways. The ability to isolate purified RIN1 should facilitate the further characterization of specific binding properties. Of particular interest will be the examination of how interaction affinities are regulated through posttranslational modifications that may also be cell type specific.

[27] F. Finney and D. Herrera, *Methods Enzymol.* **255**, 310 (1995).

# [11] Ras and Rap1 Interaction with AF-6 Effector Target

*By* BENJAMIN BOETTNER, CHRISTIAN HERRMANN, and LINDA VAN AELST

## Introduction

The Rap types of small GTPases are members of the Ras superfamily and are the molecules that show the most identity with the oncogenic Ras proteins. Whereas the interaction of activated Ras proteins with their downstream effectors Raf, Ral guanine nucleotide dissociation stimulator (RalGDS), and phosphatidylinositol 3-kinase (PI3K) led to a fairly defined


METHODS IN ENZYMOLOGY, VOL. 333
0076-6879/01 $35.00

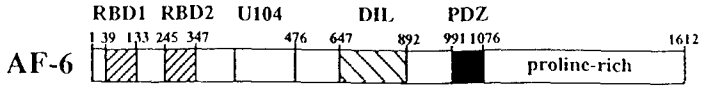



FIG. 1. Schematic representation of AF-6.

picture, the role of Rap1 is as yet poorly understood.[1] Besides its ability to bind to Raf and RalGDS, two-hybrid and *in vitro* experiments suggest that still another molecule, namely AF-6 ALL1-fused gene on chromosome 6, might serve as a relevant target. Both two-hybrid and kinetic studies suggest a strength of interaction that exceeds the one exerted by Ras–AF-6 complexes, which originally led to the identification of AF-6.[2,3] AF-6 has also been described as a fusion partner for ALL1 in acute lymphoblastic leukemias.[4] The AF-6 protein contains a combination of interesting homology regions.[5] At its very $NH_2$ terminus reside two putative Ras/Rap 1-binding motifs, followed by U104 and DIL motifs, domains that are found in the head portions of microtubule- and actin-based motor proteins, respectively. Located further to the COOH terminus is a PDZ domain succeeded by proline-rich clusters, which may function as docking sites for other molecules (Fig. 1).

In this chapter, we outline the methods that allowed us to investigate the physical interaction between Ras/Rap1 and AF-6, namely two-hybrid analyses, kinetic and thermodynamic studies, and *in vivo* studies utilizing retrovirally engineered cell lines.

AF-6 resides in cell–cell adhesion complexes and could provide the molecular link between the activity of Ras/Rap1 proteins and their effects on intercellular adhesion.

## Use of Yeast Two-Hybrid System to Evaluate Ras/Rap1 Interaction with AF-6

### *Principle*

One approach we took to examine the interaction between Ras/Rap1 and AF-6 consists of a two-hybrid interaction trap assay. This system is a

[1] J. L. Bos, *EMBO J.* **17**, 6776 (1998).
[2] L. Van Aelst, M. A. White, and M. H. Wigler, *Cold Spring Harbor Symp. Quant. Biol.* **59**, 181 (1994).
[3] M. Kuriyama, N. Harada, S. Kuroda, T. Yamamoto, M. Nakafuku, A. Iwamatsu, D. Yamamoto, R. Prasad, C. Croce, E. Canaani, and K. Kaibuchi, *J. Biol. Chem.* **12**, 607 (1996).
[4] R. Prasad, Y. Gu, H. Alder, T. Nakamura, O. Canaani, H. Saito, K. Huebner, R. P. Gale, P. C. Nowell, K. Kuriyama, Y. Miyazaki, C. M. Croce, and E. Canaani, *Cancer Res.* **53**, 5624 (1993).
[5] C. P. Ponting and D. R. Benjamin, *Trends Biochem. Sci.* **21**, 422 (1996).

genetic method that allows the determination of physical complexes between two proteins within yeast cells.[6] These proteins are expressed as hybrid proteins, one fused to a DNA-binding domain and the other fused to a transcription-activating domain. If the two proteins associate, a functional transcription factor is reconstituted and a reporter gene is transcribed. Several versions of the two-hybrid system exist; they commonly involve DNA-binding domains derived from Gal4 (GBD) or LexA (LBD) and activation domains from Gal4 (GAD) or VP16 transcriptional activators.[6,7] Numerous yeast strains with different reporter genes for both systems have been constructed, as well as variations of the original two-hybrid system, including a reverse one- and two-hybrid system, and a three-hybrid system. The more recently constructed yeast strains, with multiple reporter genes harboring independent promoters, offer a major advantage because they increase specificity, thus limiting false positives. A detailed description can be found in Vidal and Legrain[8] and in Brent and Finley.[9] The choice of using either the Gal4- or LexA-based system will be dependent on the nature of the protein of interest.[10] In our studies directed to assess the interaction between Ras/Rap1 and AF-6, we found that both Gal4- and LexA-based systems can be used. The use of the LexA-based system is presented here. The application of the two-hybrid system allowed us to assess whether the binding of AF-6 requires Ras/Rap1 to be in a GTP-bound state, to map the minimum domain of AF-6 required for interaction, and to compare binding profiles between Ras, Rap1, AF-6, and other Ras/Rap1 targets.

### *Materials*

*Yeast Strain.* The yeast strain L40 (*Mat***a** *his3Δ200 trp1-901 leu2-3,112 ade2*) containing *HIS3* and *lacZ* as reporter genes is used for the LexA-based system.[7]

*Media.* YPD medium contains 10 g of yeast extract, 20 g of Bacto-Peptone (Difco, Detroit, MI), 2% (w/v) glucose, and 20 g of agar for plates, per liter. DO-Leu-Trp and DO-Leu-Trp-His media contain Bacto-Yeast nitrogen base without amino acids (0.67%, w/v), dropout mix (0.2%, w/v), and Bacto-Agar (2%, w/v). Dropout mix is a combination of all essential amino acids minus the appropriate supplement.[10]

[6] C. T. Chien, P. L. Bartel, R. Sternglanz, and S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991).
[7] A. B. Vojtek, S. M. Hollenberg, and J. A. Cooper, *Cell* **74**, 205 (1993).
[8] M. Vidal and P. Legrain, *Nucleic Acids Res.* **27**, 919 (1999).
[9] R. Brent and R. L. Finley, Jr., *Annu. Rev. Genet.* **31**, 663 (1997).
[10] L. Van Aelst, *Methods Mol. Biol.* **84**, 201 (1998).

*Solutions*

Lithium acetate/TE (0.1 *M*, pH 7.5): Combine 0.1 *M* lithium acetate (Sigma, St. Louis, MO), 10 m*M* Tris–HCl (pH 7.5), and 1 m*M* EDTA

Polyethylene glycol (PEG) 3300, 40% (w/v) in 0.1 *M* lithium acetate

Z buffer: Combine $Na_2HPO_4 \cdot 7H_2O$ (16.1 g/liter), $NaH_2PO_4 \cdot H_2O$ (5.5 g/liter), KCl (0.75 g/liter), $MgSO_4 \cdot 7H_2O$ (0.246 g/liter); adjust to pH 7.0 and autoclave.

X-Gal stock solution: Dissolve 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim, Indianapolis, IN) in 1 ml of *N,N*-dimethylformamide. Store at −20° in the dark.

Z buffer/X-Gal solution: To 100 ml of Z buffer add 270 μl of 2-mercaptoethanol and 1 ml of X-Gal stock solution

*Plasmids and Constructs.* For the construction of pGAD AF-6N, pGAD AF-6-RBD1, and pGAD AF-6-RBD2: AF-6N (amino acids 1–368) is polymerase chain reaction (PCR) amplified with oligonucleotides AF6N-5′ *Sal*I (5′-GGGACGTCGACTCTCGGCGGGCGGCCGTGACGAG-3′) and AF6N-3′*Xho*I (5′-CGGCAGCTCGAGCTATCTCTCCTTTCCCTTGGGTGT-3′) and inserted into *Xho*I-digested pGAD1318 plasmid, which is a derivative of pGADGH.[10] AF-6-RBD1 (amino acids 1–140) is amplified with AF6N-5′ *Sal*I and AF6RBD1-3′ *Xho*I (5′-CCGCCGCTCGAGCTACTTAGGAGGAATGGC-3′) as 5′ and 3′ oligonucleotides, respectively, and treated as described above. AF-6-RBD2 (amino acids 181–368) is amplified with AF6RBD2-5′BHI (5′-GGGCCGGATCCGCCATTCCTCCTAAG-3′) and AF6N-3′*Xho*I oligonucleotides and inserted into *Bam*HI–*Xho*I-digested pGAD1318 vector. For the construction of pGAD PI3Kδ-RBD, PI3Kδ-RBD is PCR amplified using 5′ oligonucleotide PI3Kδ RBD5′ *Bam*HI (5′-CGGCGCGGATCCATGGCCAAGATGTGCCAATTCTGC-3′) and 3′ oligonucleotide PI3Kδ RBD3′ *Sal*I (5′-GCCGACGTCGACCTAGTTGCTCTGCTCATCCCG-3′), and digested with *Bam*HI and *Sal*I restriction endonucleases prior to ligation into *Bam*HI–*Xho*I-cut pGAD1318. pGAD1318 RalGDS-RBD is obtained in a yeast two-hybrid screen, using LBD Ha-RasV12 as bait and a Jurkat cDNA library cloned in pGAD1318. The plasmids pGADGH cRafN, LBD RasV12, and LBD RasN17 have been previously described.[7,11] For the LBD Rap 1E63 and Rap1N17 constructs, the cDNAs are PCR amplified with pZip-EE-Rap1E63 (obtained from B. Knudsen, Cornell University, Ithaca, NY) and pGTB9 Rap1N17 (from D. Broek, USC, Los Angeles, CA) as templates, and as primers 5′-ATTTATGGATCCTCTAGAATGCGTGAGTACAAGCTA-3′ and 5′-CTGACTCTCGAGCTAGAGCAGCAGACAT-

[11] L. Van Aelst, M. Barr, S. Marcus, A. Polverino, and M. Wigler, *Proc. Natl. Acad. Sci. U.S.A.* 90, 6213 (1993).

GATTT-3′ are used. The products are digested with *Bam*HI and *Xho*I and subcloned into a *Bam*HI–*Sal*I-digested pLexVJ10 plasmid.[10]

*Protocols and Results*

*Analysis of Ras/Rap1–AF-6 Interactions, Using Liquid Assay for β-Galactosidase*

As shown in Fig. 1, AF-6 harbors two predicted Ras-binding domains at its N terminus. To determine whether both domains are able to interact with Ras and Rap1, the RBD1, RBD2, and AF-6N domains of AF-6 are fused to the GAL4-activation domain of *GAL4*, whereas Ras and Rap1 mutants are fused to the LexA DNA-binding domain. In analogy to the well-characterized Ras–Raf interaction, we have also tested whether AF-6 requires Ras and Rap1 to be in the GTP-bound state. To this end we use constitutively active mutant forms (RasV12 and Rap1E63) and dominant negative mutant forms (RasN17 and Rap1N17) of Ras and Rap1. The Ras and Rap1 LDB fusion constructs are transformed together with either pGAD AF-6-RBD1, pGAD AF-6-RBD2, or pGAD AF-6N into the yeast strain L40, according to the protocol described below. Before performing the liquid β-galactosidase assay, we always first perform growth selection on selective medium (DO-Leu-Trp-His) and β-galactosidase filter assays (see below). The liquid culture assay allows us to compare and to quantify the strength of interactions.

*Yeast Transformation.* The protocol described below is a modification of the method of Ito *et al.*[12] and can be applied for both LexA- and Gal4-based systems.

1. Inoculate a single yeast colony into 10 ml of YPD and grow overnight at 30° with shaking. Transfer the overnight preculture into 100 ml of YPD and grow the yeast culture further at 30° with shaking (230 rpm) until an $OD_{600}$ of 0.5–0.8 is reached.
2. Harvest the cells by centrifugation at 1500*g* for 5 min at room temperature and wash them in 25–50 ml of 0.1 *M* lithium acetate in TE.
3. Resuspend the washed cells in 1 ml of 0.1 *M* lithium acetate in TE and incubate for 1 hr at 30° with shaking at 230 rpm. The yeast cells are now competent for transformation. One milliliter of cells allows for 10 transformations.
4. Add 100 μl of competent cells for each transformation into a 1.5-ml microcentrifuge tube.

[12] H. Ito, Y. Fukuda, K. Murata, and A. Kimura, *J. Bacteriol.* 153, 163 (1983).

5. Add the plasmid DNAs (approximately 0.5 to 2 $\mu$g) together with ~100 $\mu$g of sheared, denaturated salmon sperm DNA to the competent yeast cells and subsequently add 600 $\mu$l of sterile PEG–lithium acetate solution. Mix well by inversion.

6. Incubate at 30° for 30–60 min (shaking is not required) and heat shock for 15–30 min in a 42° water bath.

7. Pellet the cells by centrifugation for 30 sec in a microcentrifuge, remove the supernatants, and resuspend the cells in 100 $\mu$l of sterile TE.

8. Plate the yeast cells on DO-Leu-Trp medium and incubate at 30° until colonies appear.

*Liquid Culture Assay for β-Galactosidase.* The liquid culture assay for β-galactosidase provides quantitative data for the Ras/Rap1–AF-6 interaction, allowing us to compare the strength of interactions. The assay described below quantifies the β-galactosidase enzymatic activity by measuring the generation of the yellow compound *o*-nitrophenol (ONP) from the colorless substrate *o*-nitrophenyl galactoside (ONPG).

1. Inoculate single colonies from the yeast transformants in 1 ml of selective medium (DO-Leu-Trp) and grow them overnight. The next day, dilute the cells 5- to 10-fold in 5 ml of fresh DO-Leu-Trp medium and incubate them further until an $OD_{600}$ of approximately 0.8 to 1 is reached. Record the $OD_{600}$ for 1-ml samples of each culture.

2. Transfer 1-ml aliquots (in triplicate) to 12 × 75-mm polypropylene tubes and pellet the cells by centrifugation. Add 1 ml of Z buffer to the cells. Include a control with Z buffer alone.

3. Add 50 $\mu$l of $CHCl_3$ and 50 $\mu$l of 0.1% (w/v) sodium dodecyl sulfate (SDS) to the tubes and vortex vigorously for 10 sec to resuspend the cells.

4. Prewarm the samples to 30° for 5 min and then add 0.2 ml of ONPG solution to each tube. Vortex and incubate the reactions at 30°, until color develops (between 10 min and 6 hr). Stop the reaction by adding 0.5 ml of $Na_2CO_3$ (1 *M*) followed by brief vortexing. Centrifuge the samples for 10 min (3500 rpm at room temperature) and remove 1 ml of each sample to a disposable cuvette. Measure the OD at 420 nm against the blank.

5. Calculate the β-galactosidase activity by using the following equation: Activity (in Miller units) = $1000[(OD_{420} - OD_{blank})]/(tVOD_{600})$, where $t$ is time of incubation, $V$ is volume (ml) of initial cells aliquoted, and $OD_{600}$ is cell density of the culture.

The results obtained in the β-galactosidase liquid culture assay are shown in Table I. We noticed that AF-6N and the first domain (RBD1), but not the second domain (RBD2), were able to bind to Ras and Rap1. Furthermore, while both activated mutant forms of Ras and Rap1 interact

TABLE I
INTERACTION BETWEEN Ras/Rap1 AND AF-6

| LBD fusion | β-Gal activity of GAD-fused AF-6 domains (Miller units)[a] | | |
|---|---|---|---|
| | AF-6N | AF-6–RBD1 | AF-6–RBD2 |
| RasV12 | 99 ± 1.6 | 210 ± 1.8 | 0.7 ± 1.4 |
| RasN17 | 0.6 ± 1.4 | 0.9 ± 1.4 | 0.6 ± 1.1 |
| RapE63 | 120 ± 1.7 | 350 ± 1.9 | 1.2 ± 1.1 |
| RapN17 | 0.7 ± 1.7 | 0.8 ± 1.9 | 0.9 ± 1.1 |
| Lamin | 0.7 ± 1.3 | 0.8 ± 1.1 | 0.7 ± 1.5 |

[a] Data representative of a typical liquid β-galactosidase assay are shown. β-Gal was assayed with *o*-nitrophenyl-β-galactosidase as described in text. The values represent means ±SD of triplicate determination. (Reproduced from Boettner *et al.*[24] with permission of publisher.)

with AF-6, none of the dominant negative mutant forms show binding activity toward AF-6. This suggests that AF-6 binds Ras and Rap1 in their GTP-bound state. In addition, the data in Table I further indicate that the strength of interaction between Rap1 and AF-6 exceeds that exerted by Ras–AF-6 complexes. The findings that the affinity of interaction between Rap1 and AF-6 is greater than that between Ras and AF-6 are consistent with the kinetic studies described below.

*Comparison of Interactions between AF-6, Raf, RalGDS, Phosphatidylinositol 3-Kinase δ, and Ras/Rap1, Using Histidine Prototrophy Assay and Filter Assays for β–Galactosidase Activity*

We have made use of the two-hybrid system to see how AF-6 compares with c-Raf, RalGDS, and PI3Kδ in terms of its ability to bind to Ras and Rap1. LBD constructs expressing AF-6-RBD1, c-RafN, RalGDS-RBD, and PI3Kδ-RBD are cotransformed with LBD RasV12 and LBD Rap1E63, respectively, in the yeast strain L40 as described above. The transformants are subjected to histidine prototrophy and β–galactosidase filter assays for assessment of their respective interactions. In the first assay, the growth selection marker *HIS3* is used as a reporter, whereas in the latter transcriptional activity of the bacterial *lacZ* reporter is utilized.

To assay histidine prototrophy, pick individual transformants and spread as small patches on DO-Leu-Trp plates. After 2 days, the grown patches are replica plated first onto one or two DO-Leu-Trp plates to preclean the excess of yeast material and subsequently onto plates that in addition to leucine and tryptophan, also lack histidine (DO-Leu-Trp-His). Precleaning is important to avoid background growth.

To assay for activation of the *lacZ* reporter construct, the grown yeast patches (see above) are replica plated onto a Whatman (Clifton, NJ) No. 50 filter paper placed onto a DO-Leu-Trp plate and grown overnight. The filter with the yeast is placed in a container with liquid nitrogen for about 30 sec to lyse the cells, and then transferred (yeast cells facing up) in a petri dish containing a Whatman filter No. 3 presoaked in Z buffer/X-Gal solution (~2.5 ml of Z buffer/X-Gal solution per petri dish). Incubate at 30° and check periodically for the appearance of blue colonies.

As shown in Fig. 2, RasV12 is able to interact with all targets, the strongest interaction being with c-Raf. Rap1E63 shows strong interaction with AF-6 and RalGDS, but no association is observed with PI3K$\delta$ and only weak to no interaction is observed with c-Raf. This indicates that both GTPases, Ras and Rap1, use effector molecules that are only partially identical and that they exhibit differential binding profiles toward the targets listed above.

## Thermodynamic and Kinetic Characterization of Interaction between Ras/Rap1 and Ras-Binding Domain of AF-6

To study protein interactions and their biochemical characterization, large quantities of protein (in the range of milligrams) are required. Al-

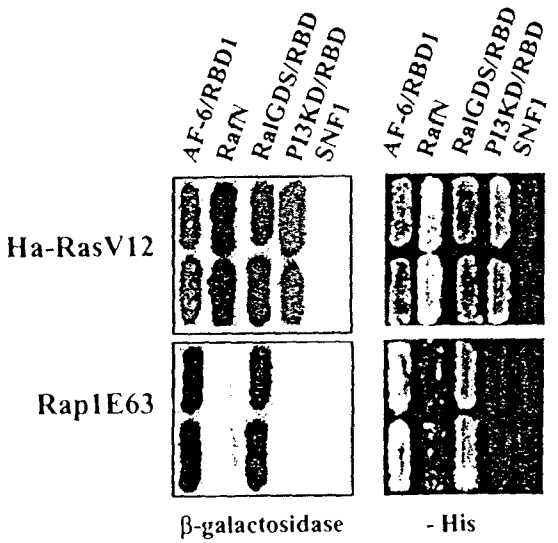



FIG. 2. Analysis of interactions between Ras/Rap1 and their effectors, using histidine prototrophy and filter assay for $\beta$-galactosidase. The LexA two-hybrid tester strain, L40, was transformed with plasmids expressing Ras and Rap1 mutants fused to LBD, and effectors of Ras and Rap1 fused to GAD. Transformants were assayed for $\beta$-galactosidase expression (*left*) and for their ability to grow on His plates. (Reproduced from Boettner *et al.*[24] with permission of publisher.)

though high yields of Ras proteins can be obtained with prokaryotic expression systems, this is usually not the case for their full-length effector proteins. Therefore, most biochemical studies are restricted to protein fragments that are soluble and can be prepared in large amounts. The Ras-binding domain (RBD) has been identified for many effectors such as Raf kinase, RalGDS, PI3K, and AF-6. The RBDs of the first three effectors are similar in size, comprising 80–90 amino acids. Despite the lack of sequence homology their three-dimensional structures are highly related, namely, they all show the ubiquitin fold.[13-15] In contrast, the RBD1 of AF-6 appears to be larger. The above-described two-hybrid studies, as well as biochemical studies,[16] indicated that the N-terminal part comprising the first 141 amino acids is a stable domain competent for strong binding to Ras and Rap1. This fragment was used for thermodynamic and kinetic investigations.

## *Methods to Quantitate Ras/Rap1 and AF-6 Interactions*

### *Fluorescence Titration*

Fluorescence is a property of many biological macromolecules that is widely used in interaction studies.[17] Two different approaches allowing fluorescence measurements are commonly employed. Either intrinsic fluorescence contributed by, for example, tryptophan residues in the protein, or extrinsic fluorescence, using chemically attached fluorescent label, may be used for monitoring binding to ligands or other proteins. Although Ras does not contain tryptophan residues, the mutant Y32W, located in switch I (effector region), shows a small decrease in fluorescence when the bound GTP is hydrolyzed to GDP.[18] Small changes in fluorescence intensity of RasY32W are observed with RalGDS–RBD (decreased fluorescence[19]) and Raf–RBD (increased fluorescence[20]). This effect is larger at lower temperatures and can in principle be used for titration experiments. However, these experiments are feasible only for RalGDS–RBD, because it contains no tryptophan residues. The high background fluorescence caused

[13] N. Nassar, G. Horn, C. Herrmann, A. Scherer, F. McCormick, and A. Wittinghofer, *Nature (London)* **375**, 554 (1995).
[14] L. Huang, F. Hofer, G. S. Martin, and S.-H. Kim, *Nat. Struct. Biol.* **5**, 422 (1998).
[15] E. H. Walker, O. Perisic, C. Ried, L. Stephens, and R. L. Williams, *Nature (London)* **402**, 313 (1999).
[16] T. Linnemann, M. Geyer, B. K. Jaitner, C. Block, H. R. Kalbitzer, A. Wittinghofer, and C. Herrmann, *J. Biol. Chem.* **274**, 13556 (1999).
[17] L. Brand and M. L. Johnson (eds.), *Methods Enzymol.* **278** (1997).
[18] K. Yamasaki, M. Shirouzu, Y. Muto, J. Fujita-Yoshigaki, H. Koide, Y. Ito, G. Kawai, S. Hattori, S. Yokoyama, S. Nishimura, and T. Miyazawa, *Biochemistry* **33**, 65 (1994).
[19] C. Herrmann, G. Horn, M. Spaargaren, and A. Wittinghofer, *J. Biol. Chem.* **271**, 6794 (1996).
[20] J. R. Sydor, M. Engelhardt, A. Wittinghofer, R. S. Goody, and C. Herrmann, *Biochemistry* **37**, 14292 (1998).

$$\begin{array}{ccc} \text{Ras}\cdot\text{mant-GppNHp} + \text{AF-6} + \text{GppNHp} & \overset{K_d}{\rightleftharpoons} & \text{Ras}\cdot\text{mant-GppNHp}\cdot\text{AF-6} + \text{GppNHp} \\ \downarrow k_1 & & \downarrow k_2 \\ \text{Ras}\cdot\text{GppNHp} + \text{AF-6} + \text{mant-GppNHp} & & \text{Ras}\cdot\text{GppNHp}\cdot\text{AF-6} + \text{mant-GppNHp} \end{array}$$

SCHEME 1.

by the tryptophan residues present in Raf–RBD (one) and in AF-6–RBD1 (two) make a direct titration impossible. Nonetheless, as we discuss below, kinetic measurements using this RasY32W mutant, together with the stopped-flow technique, allow a detailed analysis of Ras/AF-6–RBD1 complex formation.

Another method to characterize the biochemistry of Ras-like GTPases makes use of the fluorescent 2′,3′-*N*-methylanthraniloyl (mant) group attached to the ribose moiety of the nucleotide bound by the GTPases.[21,22] Binding of mant-GDP or mant-GTP to Ras or Rap1 results in a more than 2-fold increase in fluorescence. A small decrease in fluorescence intensity is observed when Raf–RBD or AF-6–RBD1 is bound.[20] As seen with the Y32W mutant, this effect diminishes with increasing temperature. However, in this case, a direct titration of Ras/Rap1–mant-GppNHp with AF-6–RBD1 is feasible with a highly accurate fluorescence detection system.[16] A broader application of the mant label is described in the following sections.

*Inhibition of Guanine Nucleotide Dissociation: GDI Assay*

As mentioned above, the dissociation of mant-nucleotides from Ras-like GTPases results in a large decrease in fluorescence intensity. It has been further observed that binding of effectors to the Ras proteins inhibits the dissociation of the bound nucleotide.[23] These features have resulted in the development of a method that allows quantification of all Ras GTPase–effector interactions. To block the GTPase activity of Ras or Rap1, the following experiments all use the nonhydrolyzable GTP analog GppNHp (guanyl-5′-yl imidodiphosphate). For example, the Ras–AF-6 interaction is described below. When Ras–mant-GppNHp is incubated together with a large excess of nonlabeled GppNHp, the latter quantitatively displaces the mant-nucleotide (Scheme 1). Because of the large excess of GppNHp, virtually no rebinding of mant-GppNHp occurs, and thus this part of the reaction is ignored in Scheme 1. Equation (1) describes the dependence of the observed dissociation rate constant of mant-GppNHp ($k_{obs}$) on the AF-6 concentration. The rapid equilibration of the Ras–AF-6 complex is

[21] H. Rensland, A. Lautwein, A. Wittinghofer, and R. S. Goody, *Biochemistry* **30**, 11181 (1991).
[22] C. Lenzen, R. H. Cool, and A. Wittinghofer, *Methods Enzymol.* **225**, 95 (1995).
[23] C. Herrmann, G. A. Martin, and A. Wittinghofer, *J. Biol. Chem.* **270**, 2901 (1995).

illustrated by kinetic studies (see below) and is a prerequisite for Eq. (1) to hold. The rate of nucleotide dissociation is measured in the presence of different AF-6 concentrations, and this essentially corresponds to a titration experiment with $k_{obs}$ as a readout. The $K_d$, describing the affinity of AF-6 for Ras, is then retrieved by fitting the values of $k_{obs}$ obtained at different AF-6 concentrations to Eq. (1).

$$k_{obs} = k_{-1} - (k_{-1} - k_{-2})\{(Ras_0 + AF\text{-}6_0 + K_d) - [(Ras_0 + AF\text{-}6_0 + K_d)^2 - 4Ras_0AF\text{-}6_0]^{1/2}\}/(2Ras_0) \quad (1)$$

where $Ras_0$ and $AF\text{-}6_0$ denote total concentrations of the Ras protein and AF-6–RBD1, respectively, and $K_d$ is the equilibrium dissociation constant of the complex.

*Nucleotide Loading.* The synthesis of the fluorescent nonhydrolyzable GTP analog, mant-GppNHp, has been described.[22] To exchange the GTPase-bound GDP for the nonhydrolyzable GTP analog, the Ras protein (20 mg/ml) is incubated for 1 hr at 20° with alkaline phosphatase (2 U/mg) and a 2-fold excess of mant-GppNHp in the presence of 200 m*M* ammonium sulfate. The alkaline phosphatase hydrolyzes GDP, thereby quantitatively loading mant-GppNHp (or GppNHp) onto Ras. In the case of Rap1, 10 m*M* EDTA is also included. Furthermore, excess nucleotide and salt are removed by gel filtration and the mant-GppNHp-bound Ras protein is thereby transferred into the desired buffer.

*GDI Assay.* The GDI assay is performed by thermostatting 50 n*M* Ras · mant-GppNHp and varying concentrations of AF-6–RBD1. This is done in fluorescence cuvettes at 37°. The buffer contains 5 m*M* $MgCl_2$, 20 m*M* Tris (pH 7.5), and NaCl, to set the desired ionic strength. To obtain reliable results, 8 to 12 different AF-6–RBD1 concentrations ranging below, near, and up to 5 times the $K_d$ value should be tested. The dissociation (displacement) of the mant-nucleotide is initiated by the addition of 100 $\mu M$ GppNHp, and the fluorescence is excited at 360 nm and monitored at 450 nm. An exponential decay curve is fitted to the fluorescence time trace, yielding $k_{obs}$. These data are plotted versus the effector concentration, and the $K_d$ value is obtained from the fit according to Eq. (1).

The GDI assay has been applied to many different Ras proteins, their mutant variants, and different effectors such as Raf, RalGDS, and AF-6.[16,19,23] For AF-6–RBD1, a $K_d$ value of 0.25 $\mu M$ was reported for Rap1A, which binds 12 times more strongly than Ras ($K_d$ of 3 $\mu M$[16]). These data are consistent with the two-hybrid results described above.

### *Stopped-Flow Technique*

The dynamics of Ras/Rap1 and AF-6 interactions have been investigated by means of stopped flow. In this technique, two solutions containing

Rap1 · mant-GppNHp and AF-6–RBD1, for example, are rapidly mixed (within 1 msec) and the time trace of the fluorescence change due to association of the proteins is recorded. In contrast to equilibrium fluorescence titration, the stopped-flow technique can be applied even when small changes in fluorescence intensity occur (<5%). In a stopped-flow experiment, this small fluorescence change is sufficient for monitoring the binding, whereas in titration experiments, it is difficult to take many readings for the accurate determination of the $K_d$ value. Stopped-flow experiments yield dissociation and association rate constants ($k_{off}$ and $k_{on}$) and from their ratio the $K_d$ value can be calculated.

As in the titration experiment, the fluorescence is excited at 360 nm. However, all light emitted above 400 nm is detected with the use of a cutoff filter. The concentration of AF-6–RBD1 should be at least 5-fold in excess of Rap1 · mant-GppNHp, to fulfill pseudo first-order conditions and to allow a single-exponential fit to the recorded trace. From this fit, the obtained $k_{obs}$ is plotted versus the concentration of AF-6–RBD1. According to Eq. (2), the slope of the fitted straight line corresponds to $k_{on}$ and the intercept yields $k_{off}$.

$$k_{obs} = k_{on}[\text{AF-6–RBD1}] + k_{off} \quad (2)$$

In many cases, as with Rap1/AF-6–RBD1, $k_{off}$ is small and therefore not reliably obtained by this extrapolation. An accurate $k_{off}$ value can, however, be obtained by a displacement experiment. In this case, the Rap1 · mant-GppNHp · AF-6–RBD1 complex is placed in one syringe of the stopped-flow apparatus and is mixed with a large excess (>20-fold) of nonlabeled Rap1 · GppNHp from another syringe. Like in the GDI assay, the time trace is fitted by an exponential curve, in this case yielding $k_{off}$, the dissociation rate constant of AF-6–RBD1 and Rap1. This method has been used to characterize the Ras and Rap1 binding kinetics with AF-6–RBD1,[16] which are included in Table II. A more than 10% change in fluorescence obtained with saturating amounts of AF6–RBD1 allows comfortable detection of the fluorescence transience.

As a complement to the use of the mant-labeled fluorophore, we also used the intrinsic fluorescence of RasY32W in our stopped-flow assay. The experiments are carried out according to the evaluation strategy described above; however, in this case the fluorescence excitation is set at 290 nm and detection is through a 320-nm cutoff filter. In Fig. 3A, a typical fluorescence trace is shown, demonstrating a small fluorescence change. This is mainly because RasY32W is not sensitive to effector binding,[20] and because of the large background of AF-6–RBD1, owing to the presence of two tryptophans. To reduce the background, we use a concentration of AF-6–RBD1 no more than 5-fold in excess of RasY32W · GppNHp. Furthermore, we have chosen a low temperature, at which the change in fluores-

TABLE II
RESULTS OBTAINED BY STOPPED-FLOW EXPERIMENTS[a]

| Complex | $k_{on}(\mu M^{-1}\,sec^{-1})$ | $k_{off}(sec^{-1})$[b] | $k_{off}(sec^{-1})$[c] | $K_d(\mu M)$[d] |
|---|---|---|---|---|
| RasY32W·GppNHp[e] | 19 | 13 | 11 | 0.58 |
| Ras·mant-GppNHp[f] | 6.4 | 20.8 | 15.3 | 2.4 |
| Rap1·mant-GppNHp[f] | 11.9 | — | 2.6 | 0.22 |

[a] T. Linnemann, M. Geyer, B. K. Jaitner, C. Block, H. R. Kalbitzer, A. Wittinghofer, and C. Herrmann, *J. Biol. Chem.* **274,** 13556 (1999).
[b] Obtained from the intercept of the linear fit (see text).
[c] Obtained from displacement experiment.
[d] Calculated from $k_{off}/k_{on}$.
[e] 20 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$.
[f] 20 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, 100 m*M* NaCl.

cence is larger. In addition, NaCl is avoided in the buffer in order to have tighter binding. The observed rate constants are plotted in Fig. 4 versus the concentration of AF-6–RBD1, and the slope of the fitted straight line yields $k_{on} = 19\ \mu M^{-1}\,sec^{-1}$. However, the intercept corresponding to $k_{off} = 13\ sec^{-1}$ has an uncertainty of at least 50%. Therefore, we also performed displacement experiments, such as the typical trace shown in Fig. 3B. These experiments result in a much more reliable value of $k_{off} = 11\ sec^{-1}$.

This example emphasizes the strength of the stopped-flow technique. In addition to the kinetic constants, the $K_d$ value can also be obtained using $K_d = k_{off}/k_{on}$ with only a 1.5% change in fluorescence intensity. This small change in fluorescence would not allow determination of the affinity by titration experiments. In Table II, the results obtained from the RasY32W mutant and mant labeling are compared. The results of the two systems agree well with each other, despite the different labels and the different salt concentrations used. Also, the $K_d$ values obtained by stopped flow (Ras, 2.4 $\mu M$; Rap, 0.22 $\mu M$) correlate well with the $K_d$ values derived from the GDI assay (Ras, 3 $\mu M$; Rap1, 0.25 $\mu M$).[16] Furthermore, as observed for the interaction of Ras and the effector Raf,[20] the interaction of Ras with AF-6–RBD1 is highly dynamic, with the half-life of the complex being 60 msec (at 10°).

## Establishment of Stable Ras- and Rap1-Expressing Cell Lines, Using Retroviral Gene Transfer to Study *in Vivo* Interactions between Ha-Ras/Rap1 and AF-6

### *Background and General Principles*

The generation of stable transfectants has become a common practice to investigate the functions of specific GTPases. Most cell lines expressing

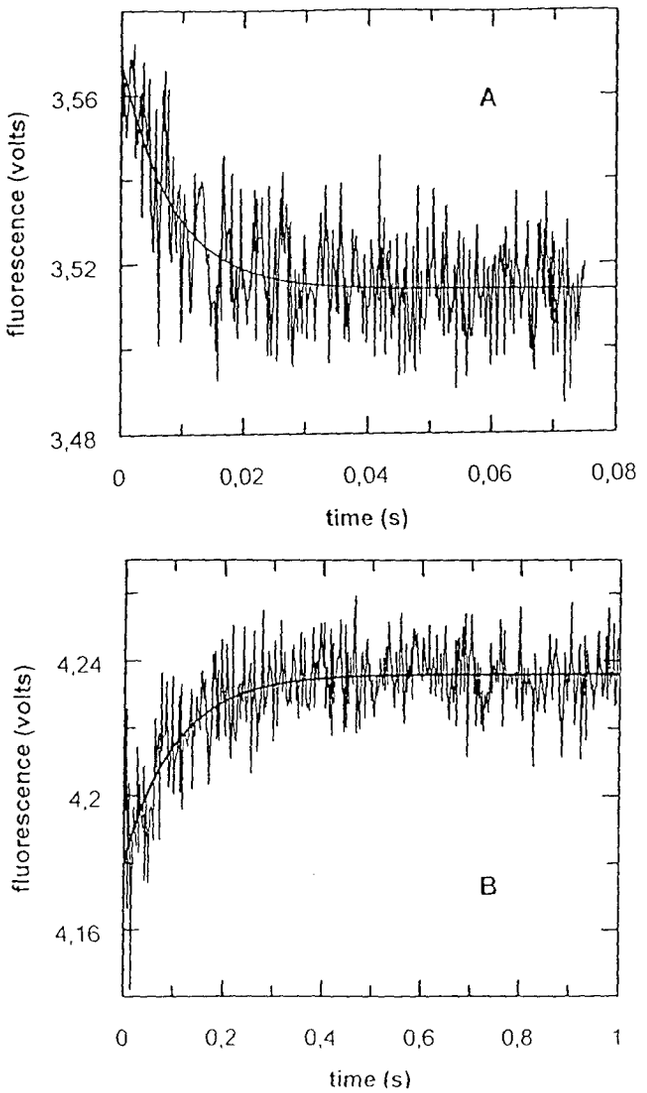



FIG. 3. Stopped-flow experiments. Fluorescence excitation was at 290 nm, and detection was through a 320 nm-cutoff filter. The buffer used consisted of 20 m*M* Tris (pH 7.5)–5 m*M* $MgCl_2$, and experiments were carried out at 10°. The graphs show the experimental trace and the exponential fit. (A) Association of 1 $\mu M$ RasY32W · GppNHp with 5 $\mu M$ AF-6–RBD1; (B) dissociation of 2 $\mu M$ RasY32W · GppNHp · AF-6–RBD1 by displacement with 40 $\mu M$ Ras · GppNHp.

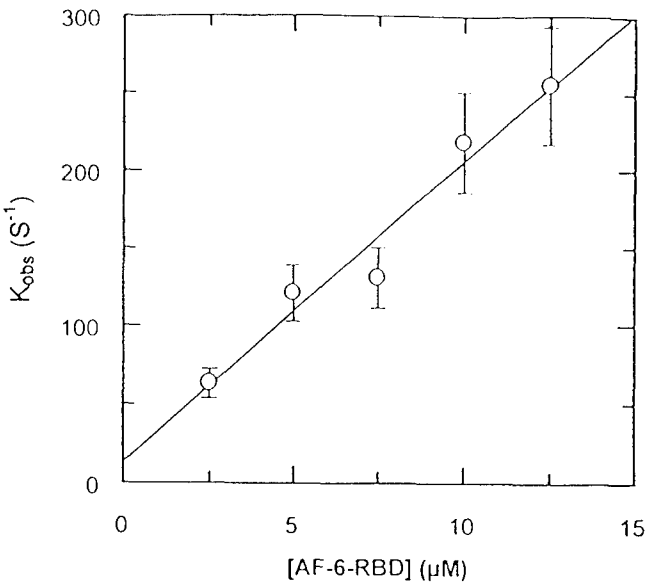



FIG. 4. Binding kinetics of RasY32W/AF-6–RBD1. $k_{obs}$ values obtained in stopped-flow experiments, as in Fig. 3A, are plotted versus AF-6–RBD1 concentrations. According to Eq. (2), the fitted straight line yields $k_{on}$ and $k_{off}$ (see Table II).

dominant active or negative mutant forms of GTPases have been generated by plasmid transfection and subsequent selection for plasmid-encoded marker genes. A more refined method for the stable introduction of Ras-type cDNAs into various acceptor cell lines is viral transduction. The efficiency of gene transfer into cell lines that are relatively intransigent to conventional transfection techniques may be considerably improved by this method. A second benefit offered by this procedure is that transduced host cells tend to lose their inserted sequences to a much lesser extent than sequences that have been conventionally transfected, when kept in culture over a long period of time. The use of packaging cell lines allows us to produce viral particles that are infectious, but cannot replicate once they have entered the host cell. Retroviruses recognize specific receptors to enter their host cells. Ecotropic receptors are present on cells of mouse and rat origin. Amphotropic receptors are present on rodent cells, as well as cells of many other species, including humans. It is possible to use an ecotropic virus in nonrodent cell lines by building the ecotropic receptor into the host cell line. Retroviruses, upon invasion, reverse transcribe virion RNA to generate linear, double-stranded DNA that integrates into the host cell genome.

To address questions of localization and interaction of the AF-6 protein in cells expressing constitutively active forms of Ras and Rap1, cell lines are generated by using retroviral vectors.[24] LinXA cells [an amphotropic packaging line provided by G. Hannon (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY)] are transfected with a pBABE[25] or pWZL vector[26] containing either the RasV12 or RapE63 insert. The virus obtained is then used to infect Madin–Darby canine kidney (MDCK) cells (nontransformed dog epithelial cells). These cell lines consist of a population of transduced cells, thus averaging out effects of retroviral integration. A detailed protocol of retroviral transfection and infection in MDCK cells is given below.

### *Materials*

*Retroviral Packaging Cell Line.* LinXA (for host cell line bearing the amphotropic receptor) and LinXE (for host cell line bearing the ecotropic receptor) packaging cell lines are a gift from G. Hannon (Cold Spring Harbor Laboratories). Alternatively, the Bosc 23 packaging cell line (for host cell line bearing the ecotropic receptor) or Bing Cak 8 (for host cell line bearing the amphotropic receptor) may be obtained from the American Type Culture Collection (ATCC, Manassas, VA) with permission from Rockefeller University (New York, NY).

*Media and Solutions for Retroviral Transfection and Infection.* The medium used for LinX packaging cells is Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) containing 10% (v/v) fetal bovine serum (FBS; HyClone). The MDCK cells are grown in DMEM containing 10% (v/v) FBS, 1% (v/v) penicillin–streptomycin, and 20 m*M* HEPES (GIBCO-BRL). Solutions required are 2.5 *M* $CaCl_2$–0.01 *M* HEPES (pH 5.5) with NaOH (sterile filter, aliquot, and store at −20°) and 2× BBS: 50 m*M* *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 m*M* NaCl, 1.5 m*M* $Na_2HPO_4$, pH ~7.00 (sterile filter, aliquot, and store at −20°). Make batches between 0.05 pH units below and above 7.00 and test which works the best. Polybrene (Sigma, St. Louis, MO) is used at a stock concentration of 8 mg/ml.

### *Retroviral Transfection and Infection of Host Cell Line*

1. Plate out $6.5 \times 10^5$ LinX packaging cells per well of a six-well plate. Allow the cells to settle and begin to adhere before returning them

[24] B. Boettner, E. Govek, J. Gross, and L. Van Aelst, *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 9064 (2000).

[25] J. P. Morgenstern and H. Land, *Nucleic Acids Res.* **18**, 3587 (1990).

[26] G. J. Hannon, P. Sun, A. Carnero, L. Y. Xie, R. Maestro, D. S. Conklin, and D. Beach, *Science* **283**, 1129 (1999).

to the incubator (approximately 10 min). Incubate overnight at 37°, 5% $CO_2$.

2. When the cells have reached approximately 70% confluency, change the medium gently (cells tend to lift easily) and incubate between 1 and 4 hr at 37°C, 5% $CO_2$.

3. Aliquot the DNA into a sterile 1.5-ml Eppendorf tube. It is best to do an initial titration using a β-galactosidase (β-Gal) construct as a readout for transfection and infection efficiency in order to determine the optimal amount of DNA for each host cell type. However, 6 μg appears to work well in most cases. Dilute DNA to a total of 225 μl with sterile water. To this tube add 25 μl of 2.5 *M* $CaCl_2$–0.01 *M* HEPES (pH 5.5).

4. Next, bubble this mixture with a pasteur or 1-ml disposable plastic pipette, using a mechanical pipette aid. At the same time, add 250 μl of 2× BBS dropwise. Add 500 μl (total) to one well of a six-well plate dropwise around the plate. Incubate for 12–16 hr at 37°, 5% $CO_2$. (Two to 4% $CO_2$ is actually optimal at this step, but 5% works as well.)

5. Change the medium and incubate for 60 hr at 32°, 5% $CO_2$.

6. Remove the virus-laden medium from well and filter through a 0.45-μm pore size syringe filter. Add to one well of host cells and subsequently add Polybrene to a final concentration of 8 μg/ml. Host cells should be plated at a concentration that will give confluency, but not overcrowding, within 2 to 3 days.

7. Spin the cells for 1 hr at room temperature at 1700 rpm in a Beckman (Fullerton, CA) tabletop centrifuge.

8. Additional rounds of infection may give better infection efficiencies, depending on the packaging line and host cell type. Between one and six rounds may be necessary, as suggested by the ATCC, when using the Bosc cell packaging line from Rockefeller University. However, in most cases, one round appears to be sufficient with the LinX packaging cell line. Incubate the cells overnight at 32°, 5% $CO_2$.

9. On the next day, change the medium and incubate the cells at 37°, 5% $CO_2$ until the cells become confluent.

10. Transfer the cells to a 10-cm plate and incubate overnight at 37°, 5% $CO_2$. Add an appropriate amount of antibiotic after the transfer, but not before 48 hr after the medium was last changed. Selection may be started at the time of transfer to a large plate; however, it is best to make sure that the cell density will support viability after selection has begun. This will be dependent on host cell type and can be checked ahead of time using a β-Gal construct. Titration of appropriate antibiotics should also be done ahead of time. Generally, if puromycin is used as the selection drug, 3 days should be adequate to select out cells containing plasmid. For the generation of MDCK RasV12 and RapE63 stable cell lines, we use 10 μg/ml for selection and 5 μg/ml for maintenance. For the analysis of

RasV12- and RapE63-expressing clones, we have made use of anti-Ras and anti-Rap1 monoclonal antibodies obtained from Transduction Laboratories (Lexington, KY).

We have observed that expression of Rap1 does not disturb cell–cell adhesion, whereas cell–cell adhesion complexes in RasV12-expressing clones are disturbed. Furthermore, coimmunoprecipitation experiments using the above-described cell lines have been performed to show *in vivo* association between Ras/Rap1 and AF-6.[24]

## Conclusions

In this chapter, we have presented a spectrum of investigative approaches that served to demonstrate specific protein interactions between the Ras/Rap1 GTPases and their potential effector molecule AF-6 *in vivo* and *in vitro*. The two-hybrid analysis and the investigation of the kinetic and thermodynamic properties of Ras–AF-6 and Rap1–AF-6 complexes led to the same overall outcome, namely, that Rap1 appears to form the tightest complex with AF-6–RBD1. These data suggest a role for AF-6 as an effector in Rap1-mediated signaling. However, we cannot exclude its involvement in Ras-induced activities. Further investigations of the function of Ras/Rap1 and AF-6 in different biological contexts will shed more light on these interactions.

## Acknowledgments

We thank Eve-Ellen Govek, Arndt Schmidt, and Mingming Zhao for assistance and critical reading of this manuscript. We also thank Greg Hannon for assistance with setting up the retroviral transfection and infection assays. Our research was supported by grants from the National Institutes of Health and Department of Defense. L.V.A. is a recipient of the V Foundation and the Sidney Kimmel Foundation for Cancer Research. B.B. is a fellow of the Gesellschaft der Naturforscher der Leopoldina.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB266022
ADB260153
ADB272842
ADB283918
ADB282576
ADB282300
ADB285053
ADB262444
ADB282296
ADB258969
ADB269117
ADB283887
ADB263560
ADB262487
ADB277417
ADB285857
ADB270847
ADB283780
ADB262079
ADB279651
ADB253401
ADB264625
ADB279639
ADB263763
ADB283958
ADB262379
ADB283894
ADB283063
ADB261795
ADB263454
ADB281633
ADB283877
ADB284034
ADB283924
ADB284320
ADB284135
ADB259954
ADB258194
ADB266157
ADB279641
ADB244802
ADB257340
ADB244688
ADB283789
ADB258856
ADB270749
ADB258933
ADB265793
ADB281613
ADB284934
ADB263442
ADB284977
ADB263437
ADB265310
ADB281573
ADB250216
ADB258699
ADB274387
ADB285530