

UNCLASSIFIED

AD NUMBER
ADB253637
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 99. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St, Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 28 Aug 2002

THIS PAGE IS UNCLASSIFIED

GRANT NUMBER DAMD17-96-1-6068

TITLE: Protein Kinases in Human Breast Carcinoma

PRINCIPAL INVESTIGATOR: William G. Cance, M.D.

CONTRACTING ORGANIZATION: University of North Carolina at
Chapel Hill
Chapel Hill, North Carolina 27599-4100

REPORT DATE: July 1999

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 99). 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.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 8

20000516 108

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: DAMD17-96-1-6068

Organization: University of North Carolina at Chapel Hill

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

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

William G. Cance, M.D.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Protein Kinases in Human Breast Carcinoma			5. FUNDING NUMBERS DAMD17-96-1-6068	
6. AUTHOR(S) William G. Cance, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of North Carolina at Chapel Hill Chapel Hill, North Carolina 27599-4100			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				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Jul 99). 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) This project focuses on the biology of the Rak protein tyrosine kinase in human breast cancer. Rak is a novel tyrosine kinase our group has identified in breast cancer tissues and cell lines that has structural homology to the Src tyrosine kinase, with SH2 and SH3 domains at its amino terminus. Rak appears to be a potent growth inhibitory gene in breast cancer as induction of its expression causes cells to detach from their substratum, float in the medium, and undergo apoptosis as determined by TUNEL assay. BrdU incorporation in Rak expressing cells confirms these results. FACS analysis shows that Rak expression induces a G1 arrest that is Rb and p53 independent. Rak expression also protects cells from the effects of Taxol. Western analysis shows that Rak is expressed in a subset of human tumors, and often appears upregulated in the nodal metastases. We are also pursuing a possible interaction of Rak with the Rho family of GTPases. Through cell culture, molecular biology, and human tissue analysis we hope to further characterize the biology of this unique protein and determine its suitability as a therapeutic target.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 32	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

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.

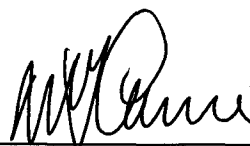
☒ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

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

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

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

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

 7/29/99

PI - Signature Date

Table of Contents

Front Cover	i
Standard Form 298	ii
Foreword	iii
Table of Contents	iv
Introduction	1
Body	2
Technical Objective 1	2
Technical Objective 2	8
Key Research Accomplishments	9
Reportable Outcomes	9
Conclusions	10
References	11
Appendices	12
Figures	13
Abstract	27

INTRODUCTION

This research project focuses on protein tyrosine kinases in human breast cancer. Specifically, we are studying the role of the novel tyrosine kinase, Rak, which our laboratory first identified several years ago (1).

Rak is a 54kd cytoplasmic tyrosine kinase that is a member of the Src family. It encodes both SH2 and SH3 domains, a kinase domain, and a bipartate nuclear localization signal. Unlike other members of the Src family, Rak lacks the myristylation signal that localizes the protein to the inner leaf of the cellular membrane. Cell fractionation studies show Rak to pellet with the nucleus. This research program has focused on the role of the Rak tyrosine kinase in human breast cancer, and we have focused on characterizing the biochemical effects of Rak expression in breast cancer cells as well as the resultant phenotypic effects to determine the utility of Rak as a therapeutic target in human breast cancer.

TECHNICAL OBJECTIVE 1

I. Characterization of the expression of Rak in human breast cancer cells.

I.A. Model Systems

In this section, we will describe the model systems that we have used to express Rak in human breast cancer cells.

I.A.1. Green fluorescence protein (GFP)-Rak (GFP-Rak).

We have constructed an expression vector which contains the coding sequence for the green fluorescence protein fused to the Rak cDNA. We have designed our constructs such that Rak is translated prior to the green fluorescence protein. The GFP-Rak vector was created in two stages. Rak was first cloned into the pMEP4 plasmid (Invitrogen) using 5'NheI and 3'XhoI sites. The Rak cDNA and Flag epitope tag were PCR amplified from pMEP4- Rak-Flag with primers created to utilize the 5' SalI and 3' KpnI sites that also are in the GFP vector multiple cloning site (Clontech). Appropriate enzymes were used to digest the fragment and vector. The vector was dephosphorylated, and ligation performed using T4 ligase. Positive colonies were screened by PCR mini-prep, and putative transformants were further screened by digesting an aliquot of purified plasmid DNA and examining for an appropriate restriction enzyme pattern. A clone passing the above two stages was submitted for automatic sequencing, and the sequence was correct.

The construct was then used for transfection into cell lines. Expression has been confirmed in multiple cell lines including COS7, BT474, MCF10, 293, SKBR3, MCF7, U2OS, and SAOS (Figures 1 and 2). The majority of the expression studies have been carried out in BT474 cells although the expression pattern is similar in all cells tested.

The expression of Rak is somewhat toxic to the breast cancer cells, as reflected in the significant floating GFP positive cells following transfection. Of the adherent cells, the GFP-Rak displays a perinuclear expression pattern. At approximately 2 days after transfection, 20 - 25% of Rak transfected cells begin to show a changing morphology. They appear to develop "arms" - filopodia type cytoplasmic extensions (see below, II.C.). After another 1-2 days, there are no longer any apparent Rak-transfected cells. It is unclear whether all the Rak transfected cells have died or whether the cells have downregulated Rak expression to such an extent that GFP is no longer visible.

~~CONFIDENTIAL~~

I.A.2. Adenoviral-Rak

In August of 1998 we submitted an Adenoviral construct containing Flag tagged Rak to the UNC viral core. When we received the first viral lysate, Rak but not the Flag tag was detected by Western blot. In conference with the directors of the viral core, they historically have had trouble with flag tag expression and suggested that we go to a HA tag.

New primers using a 5' SalI site including the HA tag and a 3' KpnI site were designed to produce an HA-tagged adenoviral construct. Expression of the clone was confirmed by western blot for both the Rak protein and HA tag. The construct was submitted in May 1999 to the UNC Viral core. We are awaiting the first trial expression lysates. If unsuccessful with adenovirus, we will pursue adeno-associated virus (AAV) system as an alternative viral expression system. Given the growth inhibition of Rak, it is conceivable that we will not be able to propagate sufficient adenovirus in the 293 cells. This problem is obviated in the adeno-associated virus system, and we are awaiting the first test of the adenoviral constructs to further address this problem.

I.A.3. Mutagenesis of the kinase domain and amino terminal deletion construct analysis

Sequence analysis shows that Rak has several putative regulatory domains including a kinase domain, SH2, and SH3 domains. We wish to generate a series of mutation and deletion constructs in the GFP-vector system to dissect the function of the various Rak domains. As pRb and Cdc2 bind to the SH3 domain, initial efforts were focused on the SH3 deletion. A double primer PCR technique (11) was first employed but was unsuccessful. We then tried single primer single strand mutagenesis with only limited success (10). We were able to generate single stranded GFP-Rak DNA with M13 phage and proceeded through the primer and synthesis stages with T4 bacterial synthetase and ligase. In mass screening we detected the presence of the SH3 deletion, but despite screening 100 possible transformed colonies, we could not obtain the desired mutant. We again returned to the double primer method, and by manipulating the PCR conditions, were able to generate the mutant. We have sequence confirmed the clone.

Initial transfections have been performed in BT474 cells. Interestingly, Rak still localizes to the perinuclear region, suggesting that the Rak SH3 domain is not required for proper localization. Furthermore, cells still form a flattened morphology and fluorescent cells have been detected with the "arms" morphology. Thus initial work suggests that the SH3 region may not be required for localization, growth inhibition, or formation of the unique "arms." This is suggestive that other domains of Rak are required for these functions. To test this, we are deleting the SH2 domain of Rak and are preparing a mutant containing a Lys262Arg mutation, which is predicted to inactivate Rak kinase activity. These mutants will be tested in a similar manner as the SH3 domain deletion (Figure 3).

~~CONFIDENTIAL~~

I.A.4. Phage display - Bacclovirus and Intein systems

In order to determine the specific molecules and sequences that bind to Rak, we are taking advantage of a technique of phage display which can isolate these sequences much more quickly than, for example, the yeast 2 hybrid system. In collaboration with Novalon Pharmaceuticals, we are producing Rak fusion proteins for phage display analysis. In this technique, Rak protein is purified and coated in wells. Specific libraries of phage, containing individual peptide sequences on their adhesion pilli are then "panned" onto wells coated with Rak protein. The phage that bind to Rak are isolated, amplified, and enriched by several additional "panning" steps. The individual phage sequences are then identified to give the peptide sequences of potential Rak binding partners. This allows us to return to a general database to identify the biologically significant partners (Figure 4).

We are currently cloning Rak into the New England BioLab's Intein vector to produce purified protein. We are also cloning into the Baculovirus system in an attempt to obtain purified protein. Once the protein is obtained, Novalon will perform the phage display. This agreement has been formally signed through the UNC Office of Technology Development.

I.B. Rak appears to co-localize in the Golgi apparatus

GFP-Rak transfected cells display green fluorescence in a perinuclear pattern reminiscent of the Golgi apparatus. A human anti-Golgi-zone monoclonal antibody (Chemicon) was obtained and used for dual immunofluorescence with the GFP-Rak construct. Microscopy revealed co-localization of GFP-Rak and Golgi. These results were further confirmed using confocal microscopy with digital analysis of images. Thus all Golgi staining lies within Rak staining, although there is some Rak staining outside of the Golgi region. Three attempts were made at disruption of the Golgi with nocodazole to see if the staining patterns still co-localized, but the technique was unsuccessful. Since Dr. Liu's lab has a primary focus of elucidating the cellular localization of Rak, we have not chosen to pursue these studies further (Figure 5).

II. Rak is Growth Inhibitory in Human Breast Cancer Cells.

II.A. Flow cytometry analyses

Based on previous work indicating that Rak binds to Cdc2 and Rb, we suspect that Rak may have an effect on the cell cycle. We performed two channel FACS analysis using GFP as a marker of Rak expression and propidium iodide as a nuclear stain. As a technical note, we used a GFP-spectrin for the control. The fixation procedure required for flow cytometric cell cycle analysis requires the use of ethanol. Ethanol fixed cells will not retain the small GFP molecule unless it is a fusion protein. Thus GFP-spectrin is useful because it has minimal effects on cell cycle but is retained during ethanol fixation.

~~CONFIDENTIAL~~
~~CONFIDENTIAL~~

The procedure to determine the cell cycle of GFP transfected cells is as follows: 1×10^6 cells are seeded onto 100mm tissue culture dishes. Cells are allowed to adhere to the plate for 24 hours. The cells are then transfected using 6ug of desired DNA and 18 ul of lipofectamine reagent. The lipofectamine/DNA complexes are allowed to incubate with the cells for 6 hours and then are washed off. The plates are overlaid with fresh full media. 30 hours later, the cells are trypsonized, ethanol fixed and suspended in 20 ug/ml propidium iodide solution in preparation for FACS analysis.

Flow cytometry of GFP-Rak and GFP-spectrin transfected cells indicate that Rak induces a modest G1 arrest: 58% (s.d.= 7) of Rak transfected cells were in G1 where as 47% (s.d.= 6) of control transfected cells were in G1 (Figure 6). Because BT474 cells are distributed predominantly in G1, it is difficult to see the small effect. Thus we used a G2 arresting drug to unveil the effects of Rak. If Rak truly arrested cells in G1, control cells would progress through G1 and arrest in G2 because of drug effects. Conversely, Rak transfected cells would arrest in G1 and be unable to respond to the drug.

The above FACS protocol was modified such that at 18 hours after transfection, drug was added to the cell media, and after incubation for 18 additional hours, the cells were harvested. Initially we used nocodazole - a microtubule destabilizing agent - but could not get consistent FACS data - cells did not respond uniformly from experiment to experiment, and controls often gave wildly varying results. Taxol - a microtubule stabilizing agent- at 1 nM for 18 hours gave excellent FACS data with consistent and predictable function of control samples. After working out technical details, we determined that Taxol causes a near complete shift of control cells towards the S and G2/M phase. Conversely, a significant proportion of GFP-Rak transfected cells remain in G1 and are thus spared from the effects of Taxol (Figure 7). Another G2 arresting agent, Colcemid (at 50 ug) has also been used and has confirmed this data (experiment done twice).

We wanted to ensure that the G1 effects of Rak were not specific to BT474 cells and thus chose to validate the results in U2OS and SAOS cells (without using drug). These lines are further useful because they differentially express Rb and p53 (Rak has been shown to bind pRb). U2OS are Rb+ p53+ and SAOS are Rb- p53-. GFP-p16 was used as a positive control for a Rb dependent G1 arrest. We demonstrated G1 prolongation in both of these cell lines which indicates that the G1 arrest of Rak is Rb and p53 independent (Figure 8 & 9). We are currently performing these flow cytometric analyses in other breast cancer cell lines.

II.B. BrdU Uptake

To further define the growth characteristics of Rak transfected cells, we utilized BrdU incorporation as a measure of cell proliferation. Approximately 30 hours after transfection, GFP-Rak, GFP-p16 control and GFP-Spectrin control cells were incubated for 4 hours with 30uM BrdU. In proliferating cells, BrdU is incorporated into newly synthesized DNA in place of thymine. After ethanol fixation and acid denaturation of the

~~CONFIDENTIAL~~

DNA an antibody can be used to detect which cells have incorporated BrdU. Unfortunately the acid denaturation also destroys the fluorescence of the GFP. Therefore, after fixation and acid denaturation, cells were dual labeled with polyclonal anti-GFP and monoclonal anti-BrdU antibodies. Goat anti-rabbit FITC conjugated secondary antibody was used to detect the GFP (FITC fluoresces green) and Goat anti-mouse Cy3 or Rhodamine conjugated secondary antibody was used to detect BrdU (Cy3 and rhodamine fluoresce red or orange).

GFP-spectrin should not affect proliferation and should show a basal level of BrdU incorporation. GFP-p16 should arrest in G1 and thus show a decreased percentage of BrdU incorporation. We expect Rak to mimic GFP-p16. Preliminary results have confirmed this hypothesis. As expected, spectrin had no effect on proliferation, as transfected cells incorporated BrdU and had an orange nucleus. In contrast, cells transfected with p16 and Rak failed to incorporate BrdU and did not have an orange nucleus, although adjacent untransfected cells continued to cycle, incorporate BrdU and thus have an orange nucleus. FACS analysis shows 39.5% of GFP-spectrin transfected cells incorporate BrdU, 25.5% of GFP-p16, and 22.7% of GFP-Rak. We are currently reproducing these results and feel that this is a clean demonstration that Rak expression inhibits S-phase entry and DNA synthesis in breast cancer cells (Figure 10).

II.C. Rak Transfected Cells Develop Filopodia-type cytoplasmic extensions

At approximately 30-40 hours post transfection, the Rak transfected breast cancer cells began to develop filopodia-type cytoplasmic extensions: "arms" phenotype. This was seen in approximately 20% of the transfected cells, but was highly reproducible both in the BT474 breast cancer cells as well as in other cell types including SK-BR-3 and BT20 breast cancer cells (Figure 2 & Figure 11 first panel).

We first wished to determine if these cells are also arrested in growth. We performed the same BrdU experiment as was described above (see Figure 10, third panel) and it is evident that Rak transfected cells with filopodial-type extensions fail to enter S-phase and incorporate BrdU. We have also examined the cytoskeletal staining in GFP-Rak transfected cells. These cells were fixed and stained with actin, vinculin, paxillin, Fak, and E-Cadherin using a rhodamine secondary. Cells were visualized using immunofluorescent microscopy. At 24 hours, Rak transfected cells demonstrated a flattened morphology and displayed filament and focal adhesion (FA) staining indistinguishable from wildtype cells. As the transfected cells progressed to develop the "arms" phenotype at 30-40 hours, they uniformly lost filamentous and FA staining as compared to wildtype. GFP control transfected cells maintained a wildtype staining pattern for filaments and FA at all time points.

In collaboration with Dr. Keith Burridge, an authority on the cytoskeleton, we hypothesize that this appearance is consistent with motile cells, and that this may represent activated Rac (note the c rather than the k). This has led us to another

~~CONFIDENTIAL~~

experimental pathway, as it would be quite significant if Rak was linked to the Rac pathway.

Rac is a member of the Rho GTPases, which are known to act as molecular switches that induce changes in the actin cytoskeleton and gene transcription in response to extracellular signals. This system participates in multiple biologic responses including morphogenesis, chemotaxis, axonal guidance, and cell cycle progression. To test our hypothesis that Rak may interact with Rac, we performed co-transfections with Rak and dominant negative Rac. If Rac is downstream of Rak, we would expect that dominant negative Rac would be incapable of forming the filopodial-like extensions. As we hypothesize, there were no "arms" formed in the breast cancer cells in this environment (Figure 11 panel 2). In addition, double transfections with Rak and control vector did not affect the development of the "arms". This suggested that Rak may be upstream of Rac and induces Rac to stimulate filopodia formation.

Next, constitutively active constructs of Rac, Rho, and CDC42 were transfected into BT474 cells (Figure 11 panel 4). Rho and CDC42 caused minimal phenotype change – the cells remained flat but compact. Active Rac caused the cells to flatten and spread radially, but there was no filopodia formation. This indicates that Rac is necessary, but not sufficient for filopodia formation in Rak-transfected cells.

Next we transfected a dominant negative construct of Rho. Surprisingly, there was a very strong "arms" phenotype. The penetrance was 85%+ and seen much earlier, at 20 hours. The same phenotype was seen with H7, an inhibitor of Rho Kinase (Figure 11 panel 3). The effects of H7 were reversible with drug removal.

Our current work is being performed in collaboration with Dr. Burridge to attempt to sort out these different pathways. A hypothesis that would fit the data and is congruent with current literature is that Rak may act as a negative regulator of Rho, which then regulates the level of activation of Rac. As Rac is more activated, the phenotype progresses from spreading radially to aggressive filopodia formation. Further work on this project will include using specific kinase inhibitors to knock out Rho function, assaying for activated Rac via a GTP binding assay, and assaying for cell motility. If true, the placement of Rak in the Rac/Rho pathway will be of great interest to breast cancer researchers. Both Rac and Rho are overexpressed in breast tumors (3). It is conceivable that Rak may regulate these proteins as breast cancer cells alter their environments and become invasive.

~~CONFIDENTIAL~~

TECHNICAL OBJECTIVE 2

I. Development and Characterization of Monoclonal Antibodies to the Rak Protein

As noted in our previous report, we have developed two monoclonal antibodies against Rak. Unfortunately, our initial monoclonal 4.65 which looked very promising for immunofluorescence, has been further characterized and found to have significant non-specific reactivity (Figure 12). Although it does have a perinuclear localization comparable to Rak's localization, extensive work with controls shows that this is non-specific staining. We have currently abandoned our experiments using monoclonal 4.65.

Our monoclonal 1.61 does recognize Rak on Western blot analyses. We have performed multiple purification and concentration techniques including ammonium sulfate cuts, protein A columns, and dialysis. We have also optimized our Western blot conditions to give the least amount of cross-reactivity. The major problems with these experiments is the low level expression of Rak. The endogenous Rak expression in breast cancer cells is quite difficult to detect and there are also significant non-specific bands on the Western blot (Figure 13).

We have used a Rak-transfected cos-7 cellular lysate as a control for our Western blots. We have done Western blots in 20 breast tumors from the San Antonio database. Eight of these expressed Rak. When we used paired tumor samples from our own tumor bank, we have detected a non-specific band close to the p54^{Rak}. We are currently characterizing the expression of Rak in additional samples to try to determine the significance of the staining (Figure 14).

Given the characterization of Rak as a growth inhibitory gene, it is not surprising that the breast cancer would express low levels of this protein. This does confound the ability for us to detect significant Rak in human breast cancers. For this reason, the majority of our effort on this project has been directed toward the characterization of the expression of Rak in breast cancer cells, as described above.

~~CONFIDENTIAL~~

KEY RESEARCH ACCOMPLISHMENTS

- ◆ Rak is growth inhibitory
- ◆ Rak produces a G1 prolongation which is p53 and Rb independent
- ◆ There is a putative interaction between Rak and the Rho family
- ◆ Rak expression causes filopodia in human breast cancer cells, resulting in decreased expression of focal adhesion components, cytoskeleton, and cadherins
- ◆ Rak localizes to the Golgi apparatus
- ◆ Rak appears to be expressed at low levels in approximately 30% of human breast cancers

REPORTABLE OUTCOMES

1. SUS abstract in New Orleans
2. Materials transfer agreement with Novalon
2. Manuscript in preparation

~~CONFIDENTIAL~~

CONCLUSIONS

Rak is a growth inhibitory gene in human breast cancer. Rak appears to cause a prolongation of the cells in G1 phase of the cell cycle, without true growth arrest. In addition, Rak causes a novel phenotype in the breast cancer cells of filopodia. Rak also appears to be in the Rac/Rho signaling pathway, although much additional experimentation is needed to conclusively confirm this association.

It is intriguing to speculate why Rak is seen in human breast cancer cells. The work with Taxol suggests that the breast cancer cells are prolonged in G1, and this may be one mechanism of resisting the apoptotic stimuli induced by Taxol. It is also possible that the filopodia is seen in the tumor cells represents the breast cancer cells becoming more motile. As the cell becomes motile, it is conceivable that it may pause in its cell cycle to allow it to move. This is a scenario which is very similar to that seen in embryonic cells. As we continue our characterizations of Rak, we hope to be able to answer these questions about this tyrosine kinase.

~~CONFIDENTIAL~~

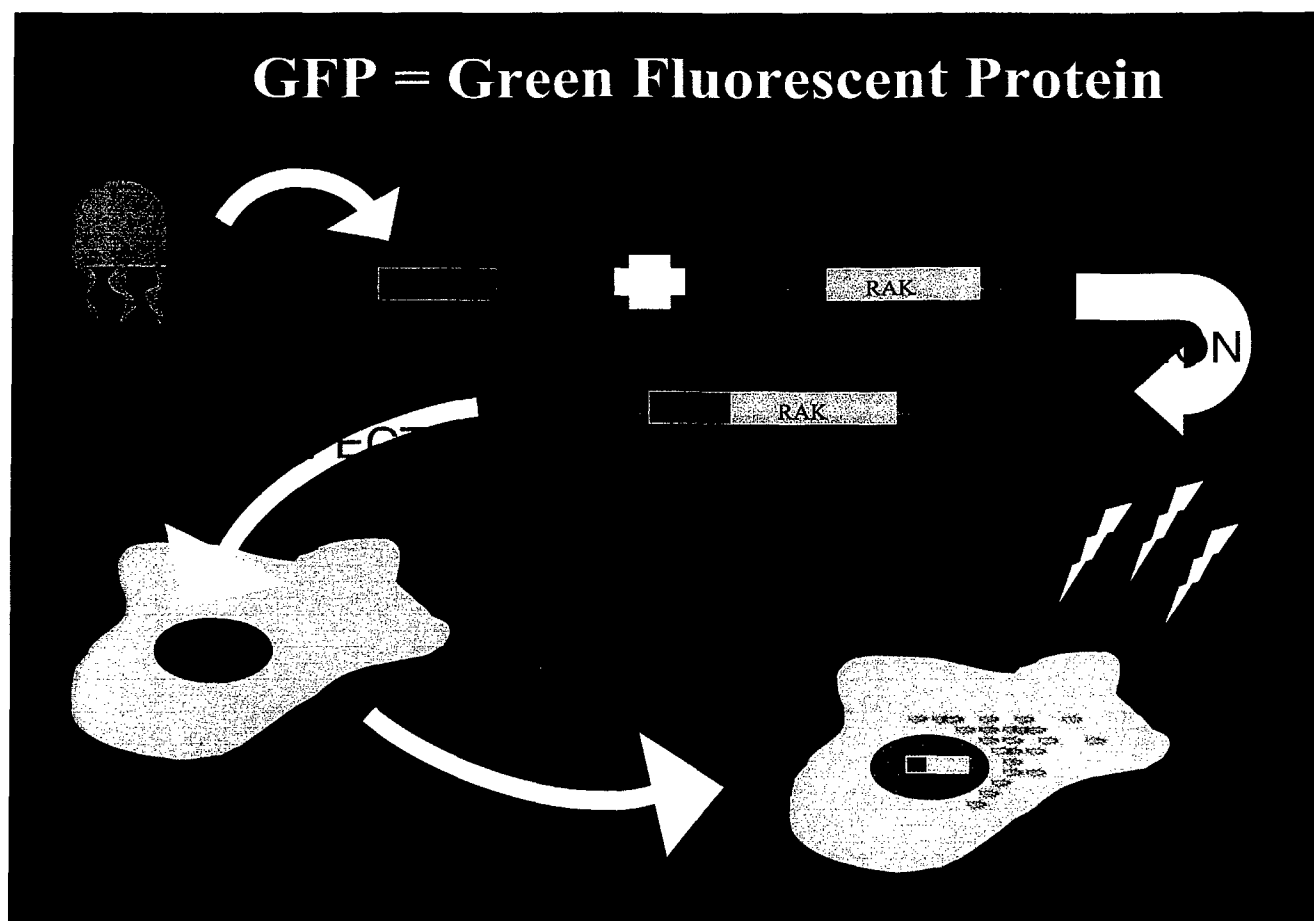
REFERENCES

1. Cance W, Craven R, Bergman M, Xu L, Alitalo K, Liu E. Rak, a Novel Nuclear Tyrosine Kinase Expressed in Epithelial Cells. *Cell Growth and Diff.* 1994;5:1347-1355.
2. Cance W, Craven R, Weiner T, Liu E. Novel Protein Kinases Expressed in Human Breast Cancer. *Int. J. Cancer.* 1993;54:571-577.
3. Fritz G, Just I, Kaina B. Rho GTPases are over-expressed in human tumors. *Int J Cancer.* 1999 May 31;81(5):682-7.
4. Hall A. Rho GTPases and the Actin Cytoskeleton. *Science.* 1998; 279:509-513.
5. Lee J. Cloning of FRK, a novel human intracellular SRC-like tyrosine kinase encoding gene. *Gene.* 1994; 138: 247-251.
6. Thuveson M. IYK, A novel intracellular protein tyrosine kinase differentially expressed in the mouse mammary gland and intestine. *Bioc. & Biop. Res. Comm.* 1995;209:582-589.
7. Vanderlaan M, Watkins B, Thomas C. Improved High-Affinity Monoclonal Antibody to Iododeoxyuridine. *Cytometry.* 1986;7:499-507.
8. Vasioukhin V. A novel intracellular epithelial cell tyrosine kinase is expressed in the skin and gastrointestinal tract. *Oncogene.* 1995; 10:349-357.
9. Yvon A, Wadsworth P, Jordan MA. Taxol Suppresses Dynamics of Individual Microtubules in Living Human Tumor Cells. *Mol. Biol. Cell.* 1999;10:947-959.
10. Promega Technical Bulletin #2195. PGEM Vector Cloning and Single-stranded DNA Generation.
11. Stratagene Technical Manual. ExSite PCR-Based Site-Directed Mutagenesis.

APPENDICES

Figures

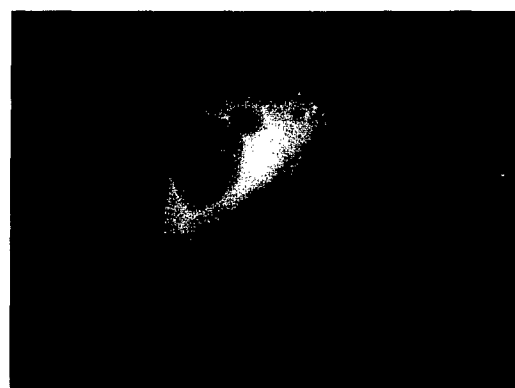
Society of University Surgeons - Abstract



Schematic of the cloning and use of the GFP fusion protein system to study gene expression.



The GFP protein alone transformed into BT474 cells. GFP alone is used as a control in all experiments. Note the homogeneous expression throughout the cell including nucleus.



Expression of GFP-Rak after transformation of the fusion construct into BT474 cells. Note the perinuclear expression pattern.

~~CONFIDENTIAL~~

FIGURE 2
GFP-Rak expression in various cell lines: note the perinuclear expression and
in several the formation of "arms"

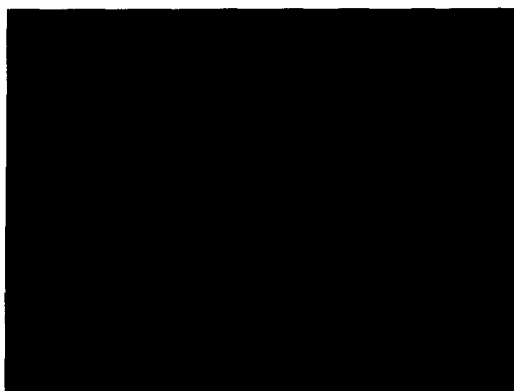
MCF7



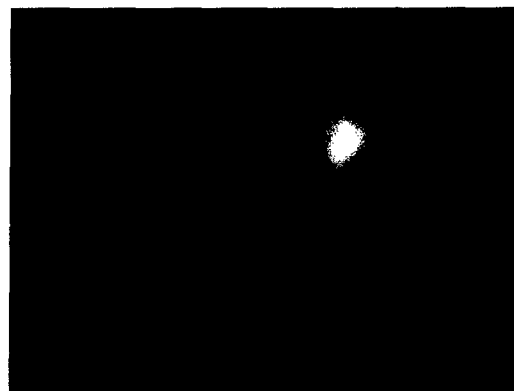
MCF7



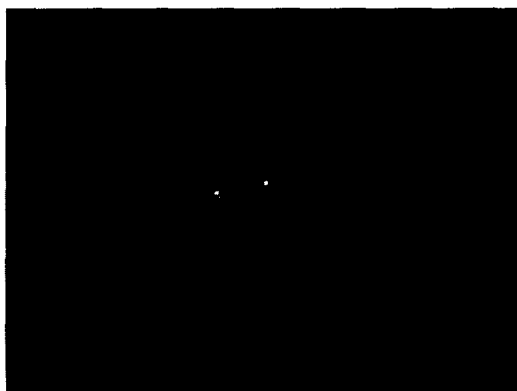
Cos7



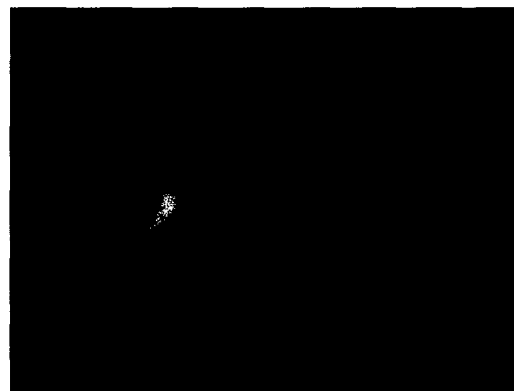
SKBr3



BT20



RD



~~CONFIDENTIAL~~

Proposed Deletion and Mutation Constructs

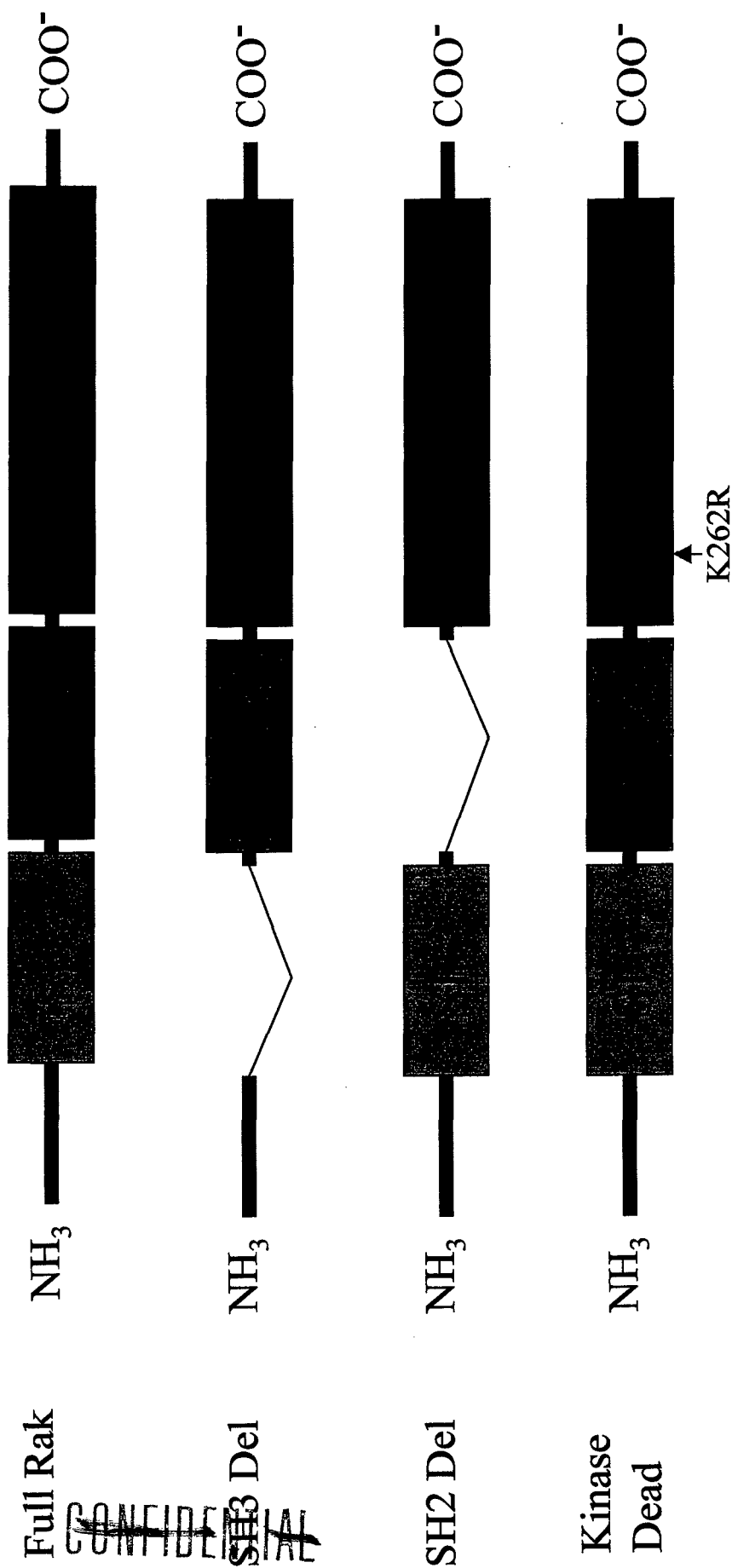


FIGURE 3

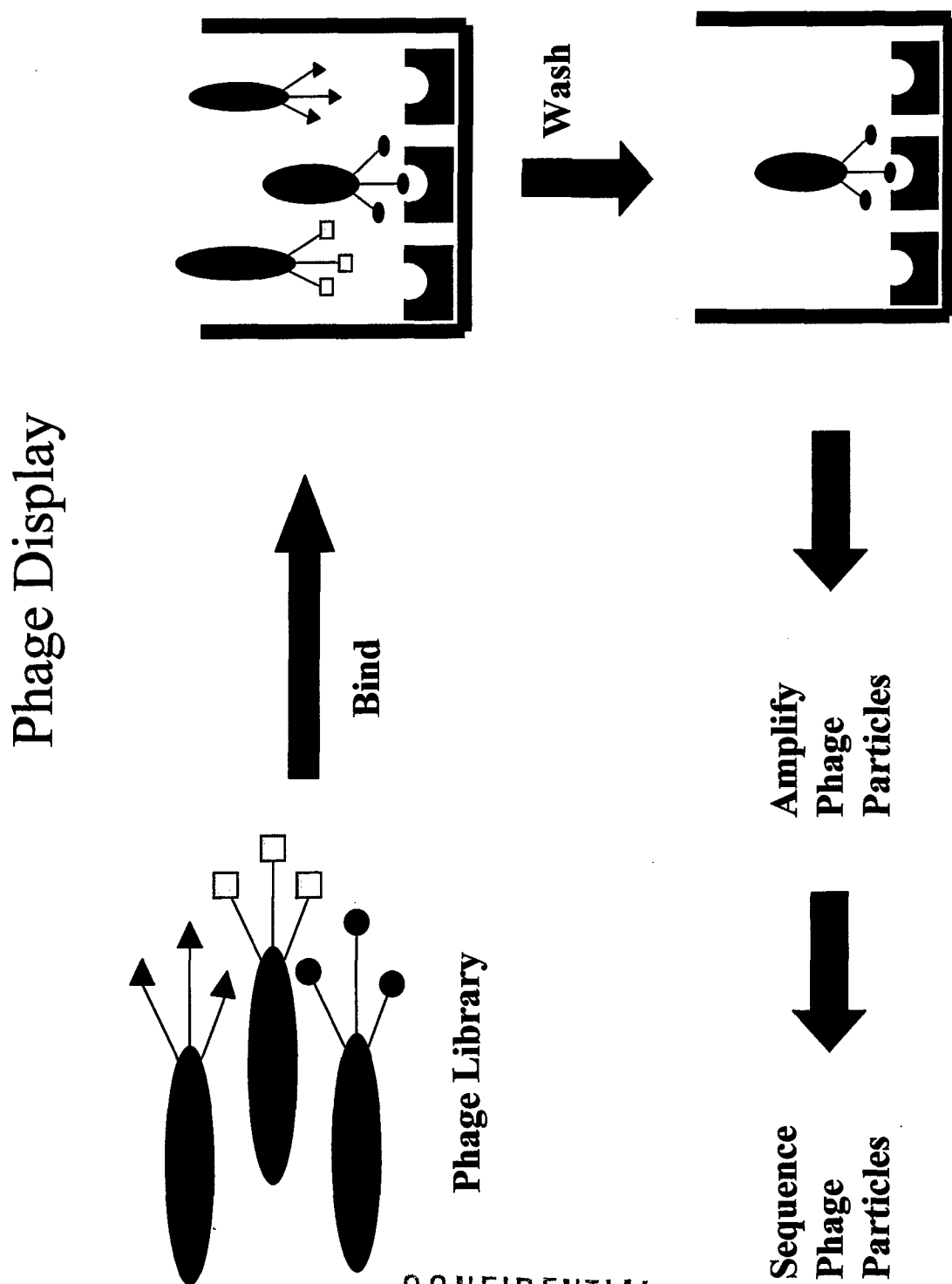
Full Rak

~~CONFIDENTIAL~~

SH3 Del

SH2 Del

Kinase
Dead



~~CONFIDENTIAL~~

Co-localization of Rak to the Golgi Apparatus



GFP-Ctl



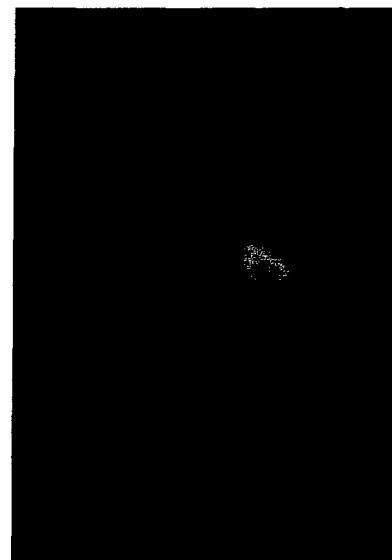
GFP-Ctl with golgi-rhodamine
secondary



Dual filter: co-localization is yellow



GFP-Rak



GFP-Rak with golgi-rhodamine
secondary



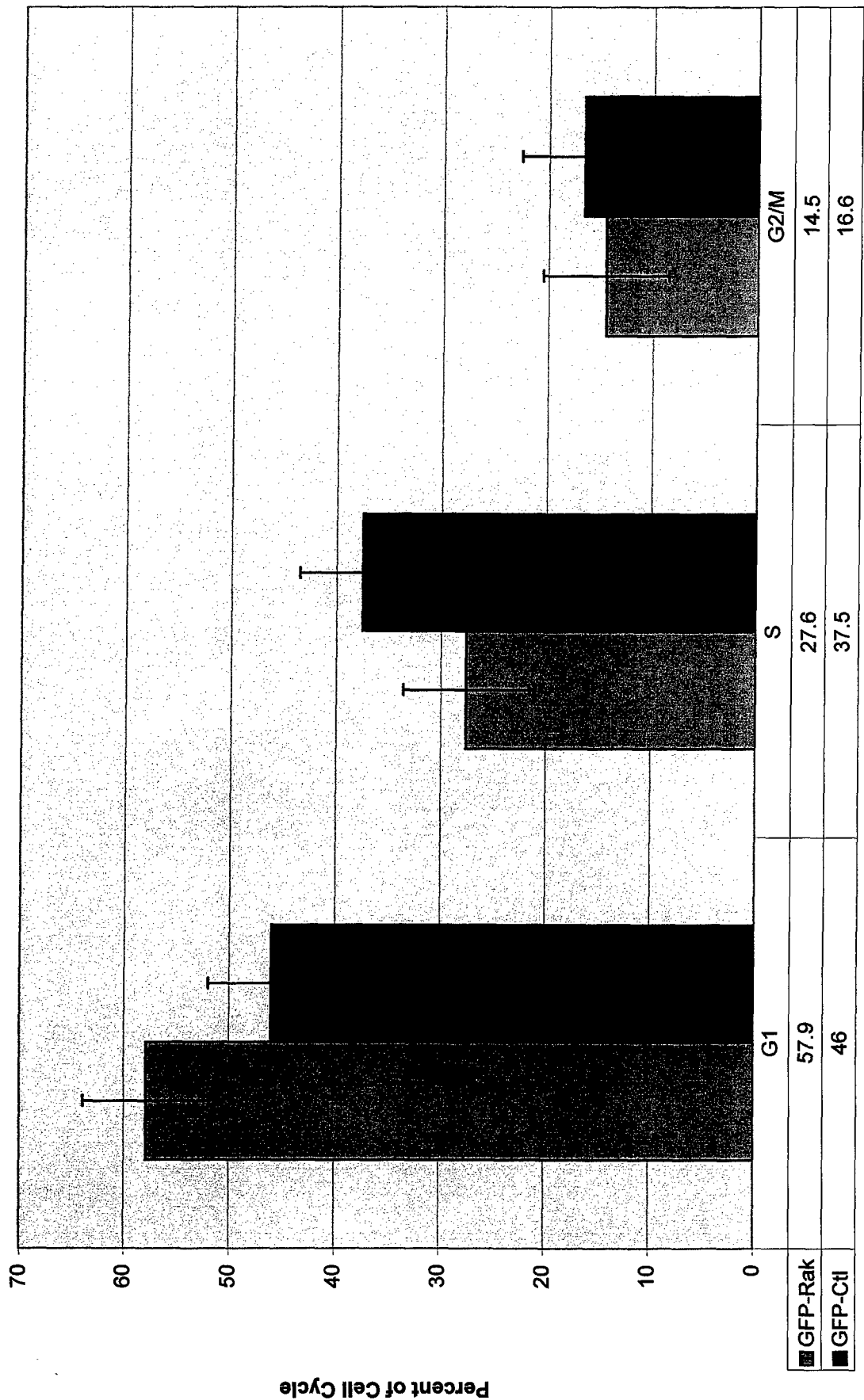
Dual filter: co-localization is yellow

FIGURE 5

~~CONFIDENTIAL~~

FIGURE 6

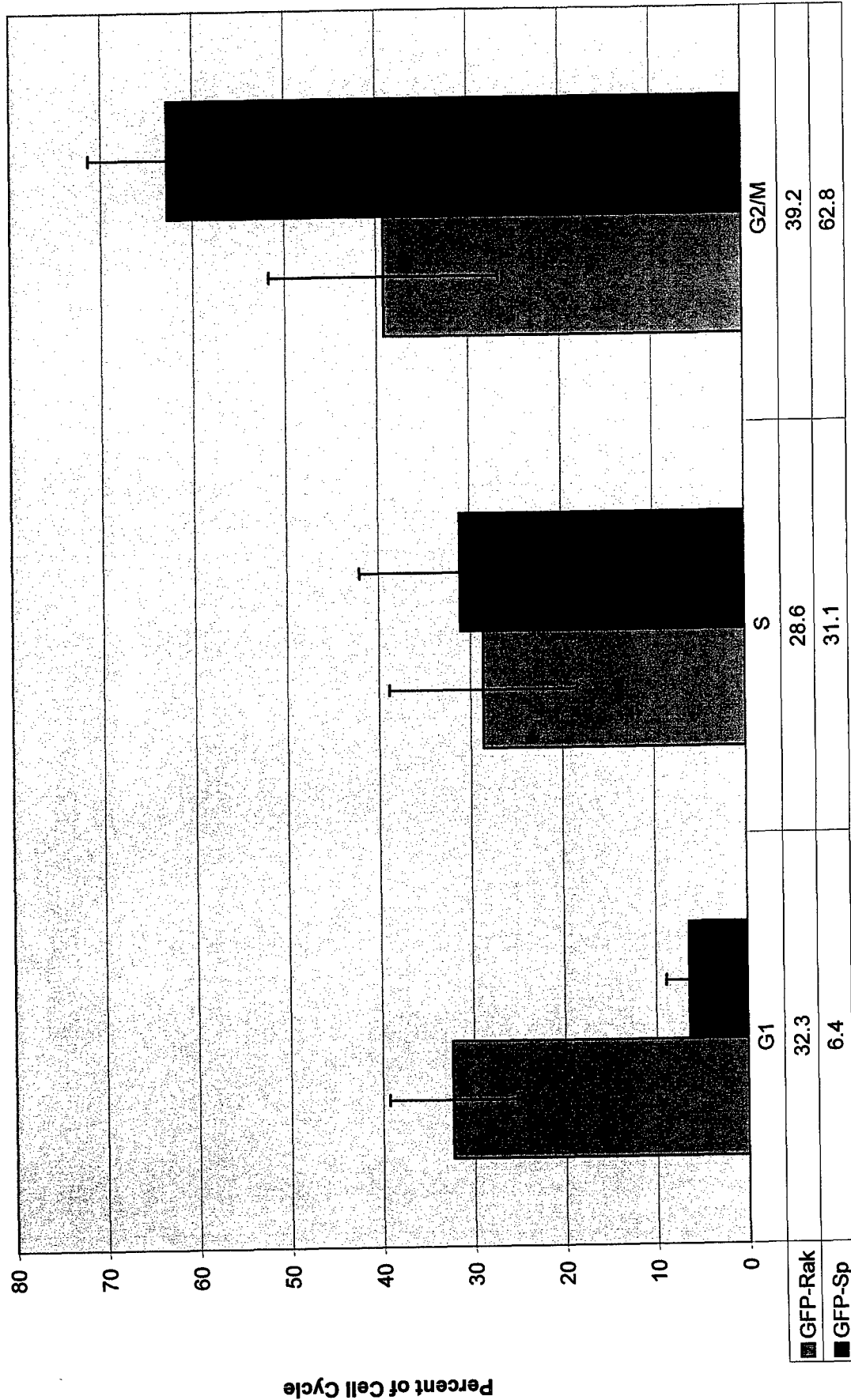
BT474 Cells Transfected with GFP-Rak



~~CONFIDENTIAL~~

FIGURE 7

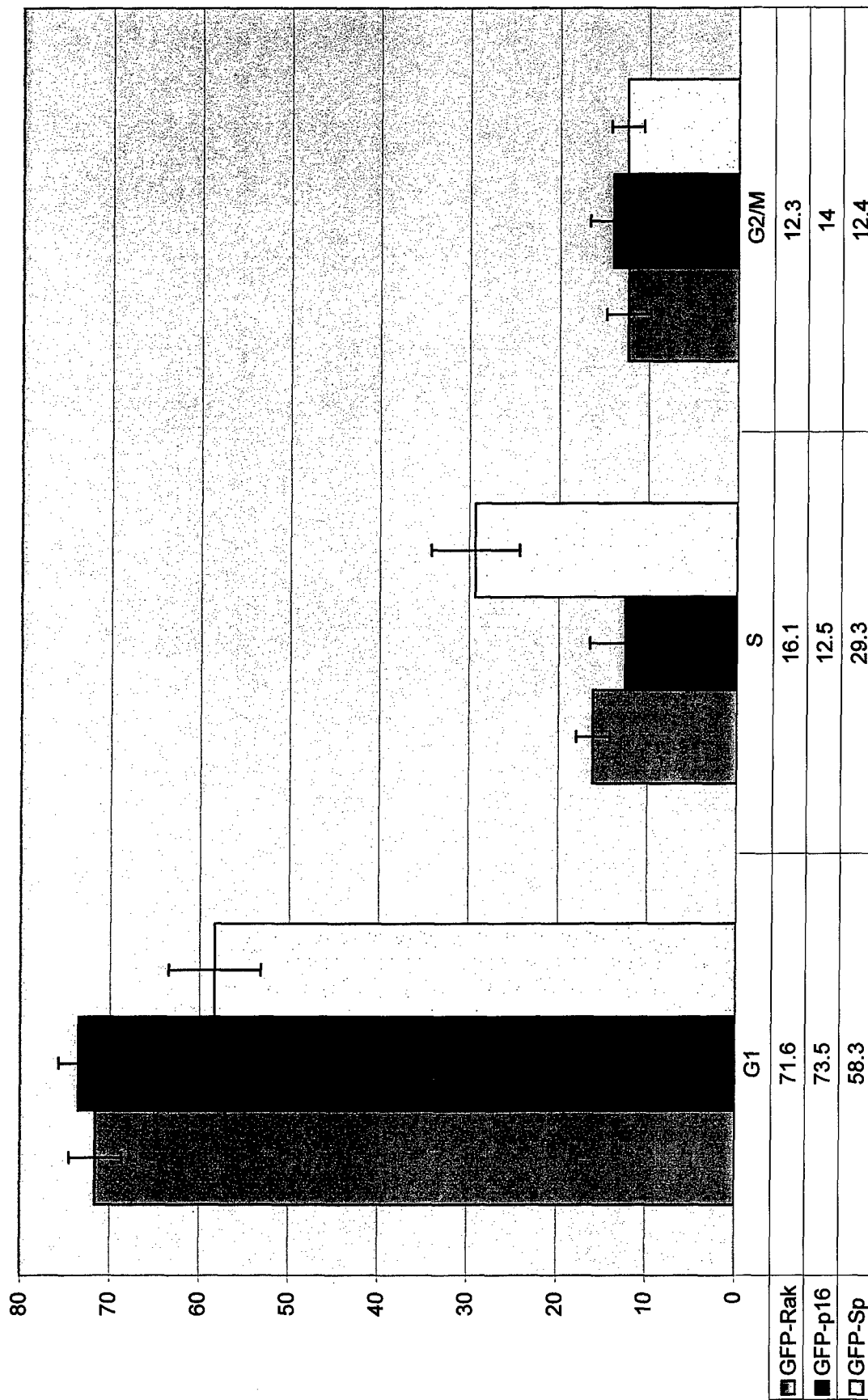
GFP-Rak with Taxol Treatment



~~CONFIDENTIAL~~

FIGURE 8

U2OS Transfected with GFP-Rak



Percent of Cell Cycle

~~CONFIDENTIAL~~

FIGURE 9

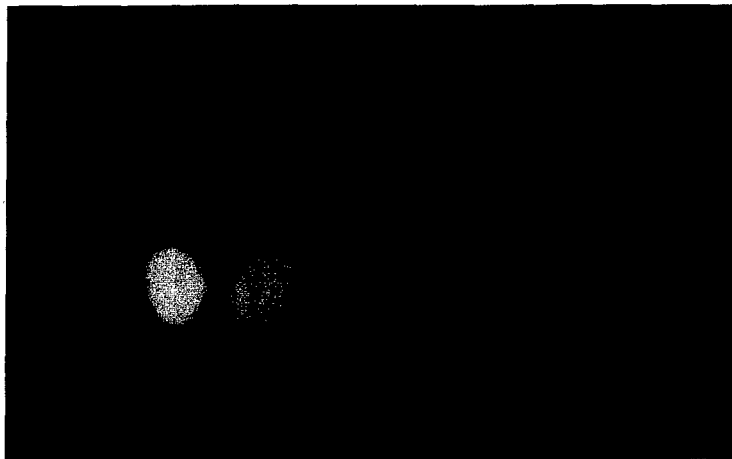
SAOS Transfected with GFP-Rak



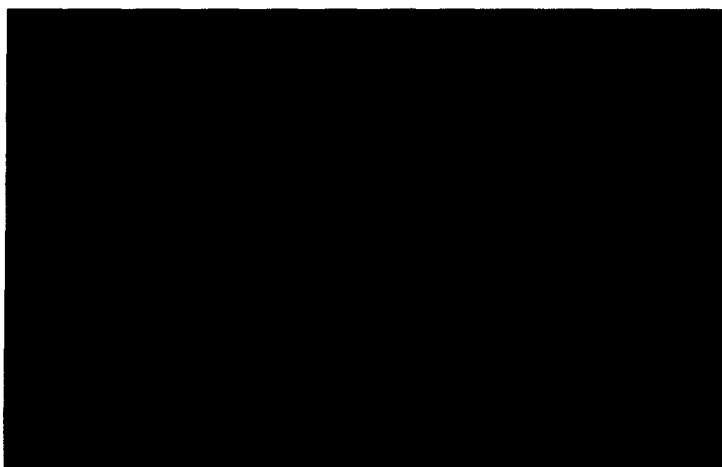
Percent of Cell Cycle

~~CONFIDENTIAL~~

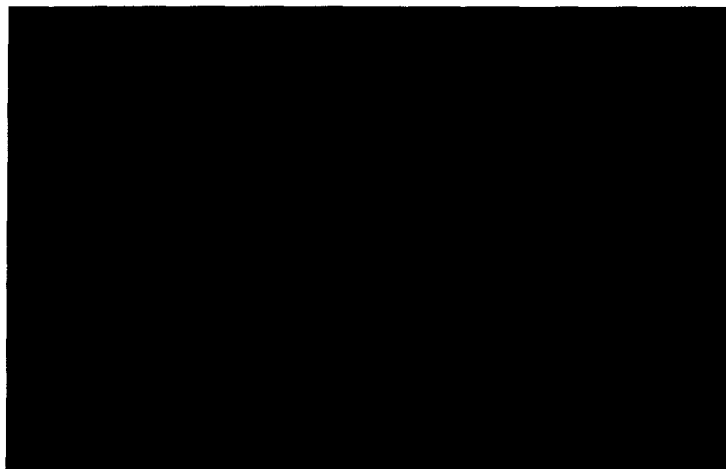
GFP-Rak with BrdU labeling to determine proliferation FIGURE 10



GFP-Spectrin transfected BT474 cells grown in 30uM BrdU and labeled using anti-BrdU Rhodamine and anti-GFP FITC. (6.7.99.12 and 6.7.99.16)



GFP-Rak transfected cells grown in 30uM BrdU and double labeled



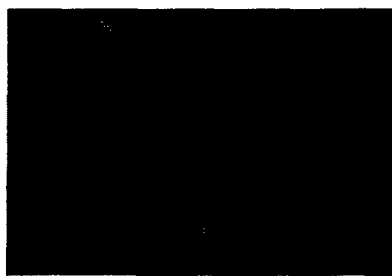
GFP-Rak transfected BT474 cells demonstrating arms phenotype and double labeled

~~CONFIDENTIAL~~

Rak may interact with the Rho GTPase pathway to affect cellular morphology



t=24h



t=40h

GFP-Rak transfected cells at 24 hours (left) show flat and compact morphology. At 40 hours (left) cells begin to develop filopodia. Nuclei are counter-stained with DAPI.

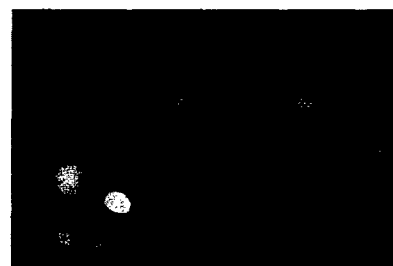


Rak/Rac-



Rak/Rac+

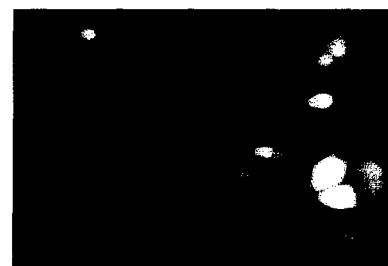
GFP-Rak and Rac Dominant Negative Constructs co-transfected (right). Rac Dominant Negative suppresses the ability of Rak to produce filopodia. GFP-Rak and Rac Constitutively Active (left) co-transfection causes cells to flatten and spread radially.



Before H7 treatment t=0



After H7 treatment t=4h



After return to Full Media t=5h

H7 is an inhibitor of Rho Kinase, which activated Rho. Treatment with H7 induces the same phenotype as Dominant Negative Rho. The effect is reversed when the drug is removed.



Rho Dominant Negative



Rho Constitutively Active

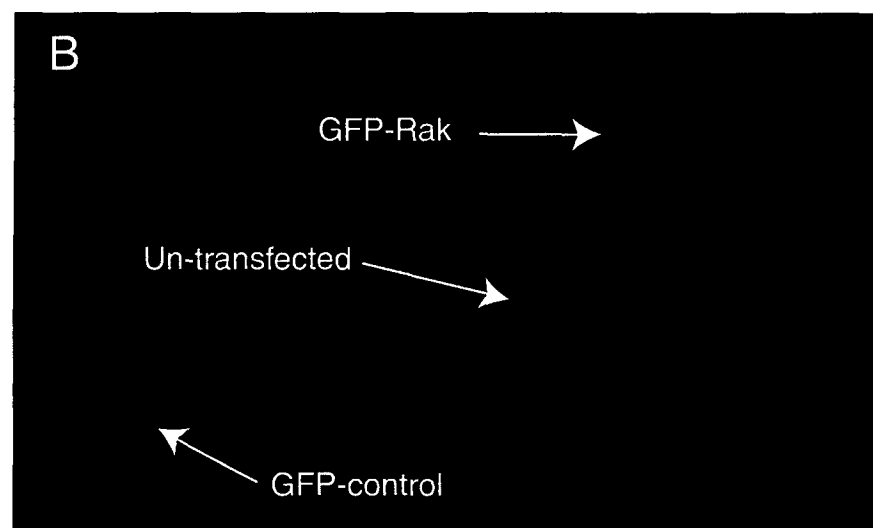
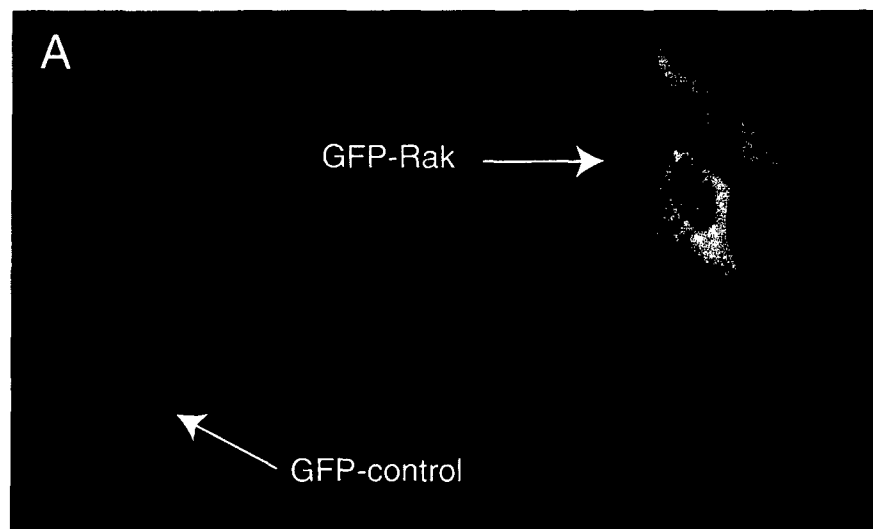


Cdc42 Constitutively Active

~~CONFIDENTIAL~~

MAb anti-Rak 4.65

Figure A shows BT474 cells transfected with both GFP-control and GFP-Rak. Figure B shows the same cells stained with MAb anti-Rak 4.65 and rhodamine secondary. All cells - transfected and un-transfected - stain. Thus, the antibody is not specific, and not useful for immunohistochemistry.



~~CONFIDENTIAL~~

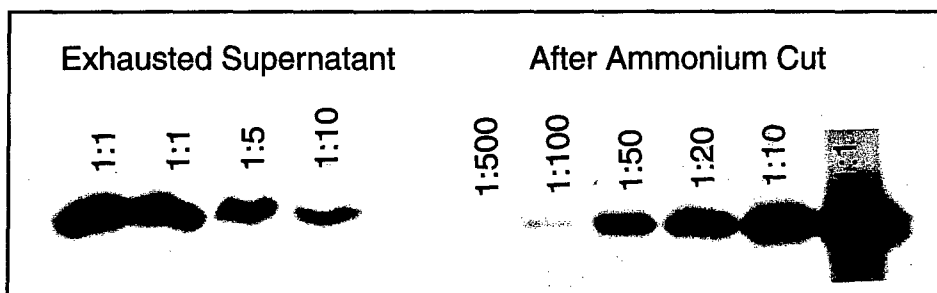


Figure 1

Ab Rak 1.61 before and after a 50% ammonium cut. The ammonium cut allows use of a much higher dilution to achieve the same band intensity thus decreasing background and conserving reagent.

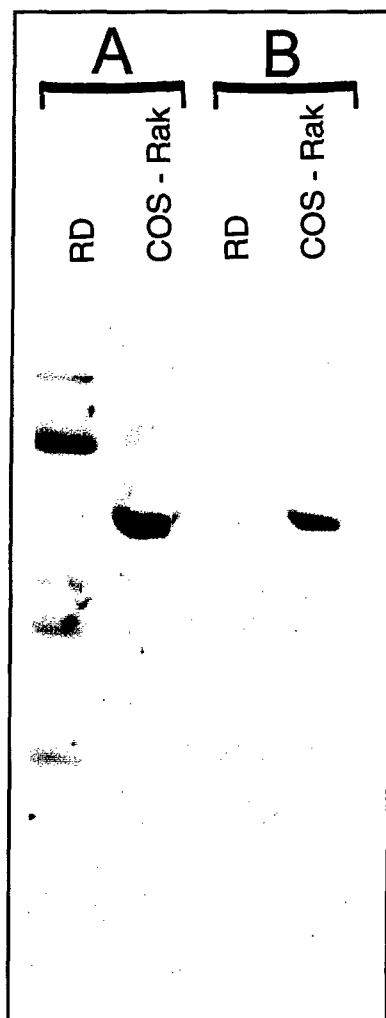


Figure 2

Ab Rak 1.61 performance under various conditions

A: Primary 1:1, TBST 0.5% Tween wash, Secondary 1:5000

B: Primary 1:3, TBST 0.5% Tween wash, Secondary 1:5000

Although using the primary antibody at a greater dilution decreases band intensity, this is compensated by achieving a cleaner blot.

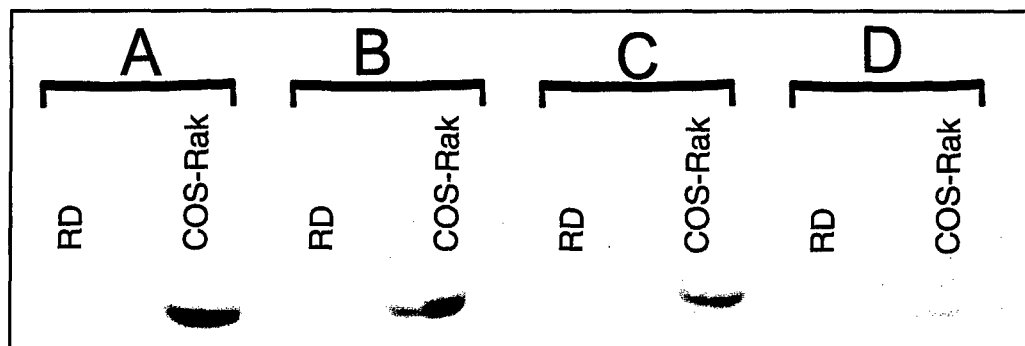


Figure 3

Ab Rak 1.61 performance under various conditions

A: Primary 1:1, TBST 0.5% wash, Secondary 1:5000

B: Primary 1:3, TBST 0.5% wash, Secondary 1:5000

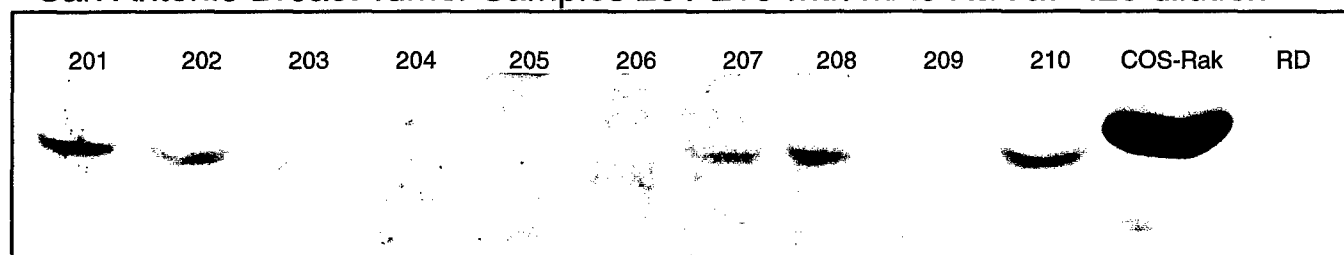
C: Primary 1:3, TBST 0.5% wash, Secondary 1:10000

D: Primary 1:3, TBST 0.5% wash, Secondary 1:10000

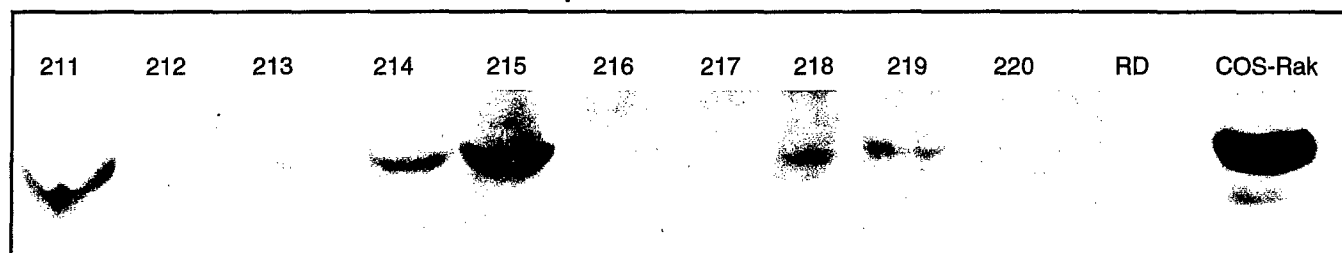
Variation to the secondary antibody diminished band intensity but had minimal effect on clarity of the blot.

Western Blots using mAb Rak 1.61 after ammonium cut

San Antonio Breast Tumor Samples 201-210 with mAb Rak at 1:20 dilution



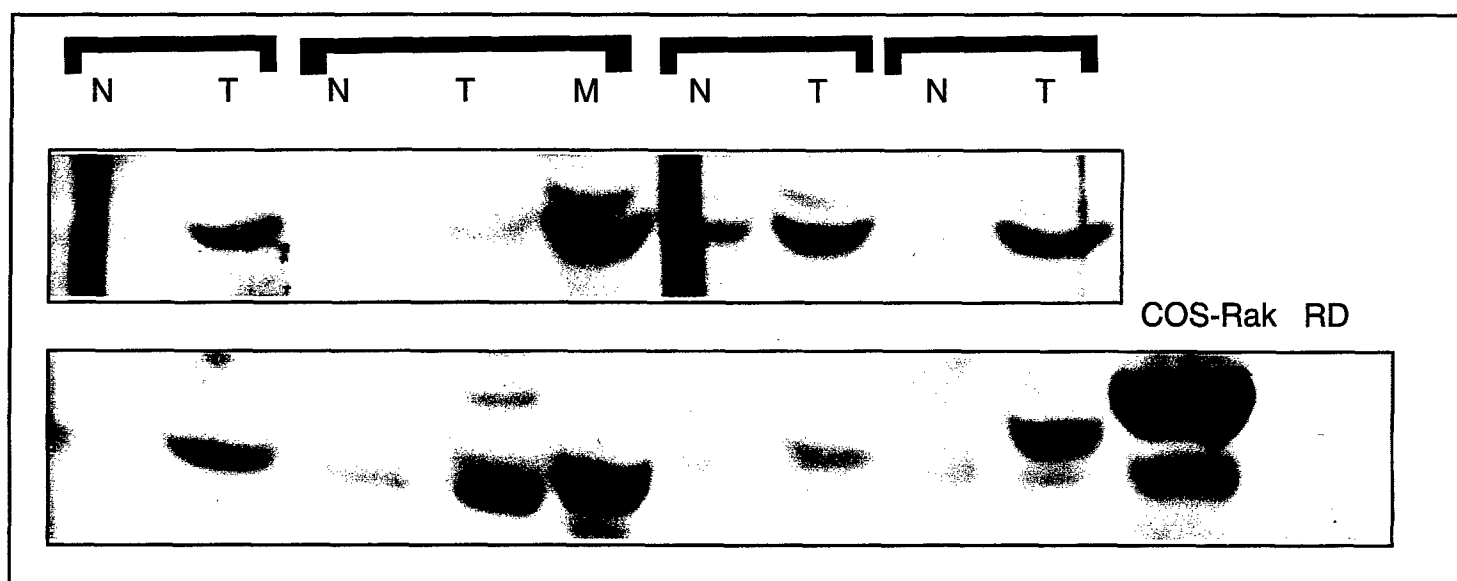
San Antonio Breast Tumor Samples 211-220 with mAb Rak at 1:20 dilution



Paired Tumor Samples with mAb Rak at 1:20 dilution

N=normal T=tumor M=nodal metastasis

Upper panel was performed initially. Lower panel displays identical samples with the addition of the positive COS-Rak control and the negative RD control



~~CONFIDENTIAL~~

THE SOCIETY OF UNIVERSITY SURGEONS
RESIDENTS' PROGRAM
February 11, 1999
New Orleans, Louisiana

PLEASE CHECK ONE (1) CATEGORY:

☐ Cardiothoracic
☐ Clinical Trials/Research Outcomes
☐ Critical Care

☐ Education
☐ Gastrointestinal
☐ Infections

☐ Nutrition
☒ Oncology
☐ Transplant

☐ Trauma
☐ Vascular
☐ Other

Authors (in order) and Institutional
Affiliation (please list initials and
Degrees of all authors):

Tanya K. Meyer, M.D.
Rolf J. Craven, Ph.D.
Elizabeth M. Roche, B.S.
William G. Cance, M.D.

Full Name of Resident Presenter:

Tanya K. Meyer, M.D.

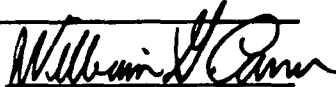
Corresponding Address of Presenter:

Department of Surgery
CB 7210
3010 Old Clinic Building
Chapel Hill, NC 27599

Tel: (919) 966-5221

Name and Address of Sponsoring SUS
Member:

William G. Cance, M.D.
Department of Surgery
CB 7210
3010 Old Clinic Building
Chapel Hill, NC 27599

Signature: 

Please note signature confirms intent
of the resident to present his/her work
at the Residents' Conference. Exception
to this rule will be at the discretion of the
Councilman-at-Large.

Submit original and three blinded copies
of abstract to:

Mary E. Samokar, Administrative Director
The Society of University Surgeons
PO Box 16549
20 Haystack Road
West Haven, CT 06516
Tel: (203) 932-0541

DEADLINE DATE:

Monday, November 16, 1998

Type Abstract in Space Below

Rak--A Novel Growth Inhibitory Tyrosine Kinase in Breast Cancer

Rak is a novel protein tyrosine kinase that was isolated from human breast cancer cells. Preliminary work suggested that ectopic expression of Rak inhibited cell growth. In this study, we have characterized the biological effects of expressing Rak in human breast cancer cells.

METHODS: Rak was cloned into the Green Fluorescent Protein expression vector (pEGFP-N1) to form a GFP-Rak fusion protein.

BT474 breast cancer cells were transiently transfected and then analyzed by dual color immunofluorescence and flow cytometry.

RESULTS: Transfection of Rak into BT474 cells demonstrated a perinuclear localization pattern. Immunofluorescent staining with an anti-Golgi antibody revealed co-localization of Rak with Golgi markers, suggesting that Rak is localized in the Golgi apparatus. In addition, the cells that expressed Rak showed a flat phenotype and appeared to be growth-arrested compared to GFP controls. Flow

cytometry results demonstrated an increased percentage of Rak transfected cells in the G₁ phase of the cell cycle, compared to control

CONCLUSION: Rak is a tyrosine kinase localized to the Golgi that inhibits human breast cancer cell growth.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 S. ...
FORT DETRICK

REPLY TO
ATTENTION OF:

MCMP-RMI-S (70-1y)

28 Aug 02

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

ADB231838
ADB240253
ADB251610
ADB275099
ADB253637
ADB261538
ADB275186
ADB264648
ADB275102
ADB241899
ADB259033
ADB266113
ADB275663
ADB254489
ADB262700
ADB276708
ADB274345
ADB274844
ADB275154
ADB275535
ADB275101
ADB275451
ADB274597
ADB273871
ADB275145
ADB274505
ADB275851
ADB274459
ADB277942
ADB277404
ADB277494
ADB277536