AD_____

GRANT NUMBER: DAMD17-94-J-4397

TITLE: The Role of HAP Kinases in Breast Cancer

PRINCIPAL INVESTIGATOR: John M. Kyriakis, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

REPORT DATE: September 1996

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (29 Aug 95-28 Aug 96)

4. TITLE AND SUBTITLE
The Role of HAP Kinases in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4397

6. AUTHOR(S)
John M. Kyriakis, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We are studying two complex signal transduction networks activated by stress and inflammatory cytokines (TNF): the SAPK and p38 pathways. The SAPKs are activated *in situ* by at least four MEKs including SEK1. p38 is activated by SEK1, MKK3 and MKK6. We are now characterizing elements upstream of these MEKs and have obtained the following results. Germinal center kinase (GCK) can potently activate the SAPKs and SEK1 in cotransfections. GCK interacts with the small GTPase Rab8. Optimal activation of the SAPKs by GCK *in situ* requires the C-terminal PEST motif. RIP, a kinase associated with the TNF receptor and Fas is a direct activator of MKK6. Finally SPRK and PAK1, two kinases upstream of the SAPKs can interact *in situ*. Our cell biological studies demonstrate that elements of the p38 pathway, including Cdc42Hs, SEK1, MKK3, MKK6 and p38 itself can each arrest cells at the G_1/S checkpoint in the cell cycle. TNF can inhibit the growth of mammary cancer cells. Our data indicate that the components which we are identifying for the SAPK and p38 pathways may mediate this growth inhibition. An understanding of these molecular players will prove important to the development of novel anticancer drugs.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer , Signal Transduction, Protein Kinases, ERKs, SAPK TNF Signaling, p38, Cell Cycle Arrest	22
	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

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	PR	- Project
G	- Grant	TA	- Task
PE	- Program Element	WU	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- DOE - See authorities.
- NASA - See Handbook NHB 2200.2.
- NTIS - Leave blank.

Block 12b. Distribution Code.

- DOD - Leave blank.
- DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- NASA - Leave blank.
- NTIS - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

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

V 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.

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.

PI - Signature _____ Date 9/25/96

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Background Pertinent to Previous and Ongoing Work

1.1.1 *General Considerations*

Cells respond to extracellular stimuli through the recruitment of signal transduction pathways. These pathways receive the external signal and act to mount a response appropriate to this signal. Signal transduction pathways which use members of the extracellular signal-regulated kinases (ERKs) have been highly conserved in evolution. These pathways consist of a central core of three protein kinases arranged in a hierarchical manner such that an ERK is activated by Tyr and Thr phosphorylation catalyzed by a mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), which is, in turn, activated by Ser/Thr phosphorylation catalyzed by a MAPK kinase-kinase (MAPKKK)(1-3).

While the ERKs and the MEKs can each be considered a single protein kinase family, at least four protein kinase families have been identified as MAPKKKs: the Raf kinases, the MEK-kinases (MEKKs), the mixed lineage kinases (MLKs, so named for their primary sequence resemblance to both Ser/Thr and Tyr kinases) as well as kinases related to the human *cot* proto oncogene (4-9). Regulation of the MAPKKKs remains largely to be elucidated, however two additional regulatory elements have been identified as upstream activators of mammalian ERK pathways. Protein kinases related to members of yeast (*S. cerevesiae*) *STE20* and *SPS1* have been shown to regulate yeast and mammalian stress-activated protein kinase pathways (1-3, 10-13). Although their mechanism of action is unclear, genetic epistasis studies of *STE20*-like kinases suggest that they are upstream of MAPKKKs (3).

In addition, all ERK pathways identified to date can be activated by small GTP binding proteins of the Ras superfamily. Thus Ras itself, by directly interacting with Raf1, can activate the mammalian mitogenic MAPK pathway (14). Likewise Rac1 and Cdc42Hs, GTPases of the Rho subgroup of Ras-like GTPases can activate mammalian stress regulated pathways, possibly through their direct interaction with and activation of p21-activated kinases (PAKs), mammalian *STE20* homologues (10,11,15). This interaction requires a distinct Cdc42Hs/Rac1 interaction binding (CRIB) domain in the amino termini of the PAKs, other *STE20* homologues and some of the MLKs (16). In addition, most PAKs have several consensus SH3 binding sites which may serve as docking modules for elements which participate in PAK signaling (1,2).

1.1.2 *Mammalian ERK pathways*

Three mammalian ERK pathways have been well characterized to date. These include a pathway regulated by mitogenic signals which employs the p42 and p44 MAPKs (17). In addition, mammalian cells employ at least two stress-regulated ERK pathways. We have discovered one such pathway, the SAPK pathway (18) The SAPKs are the principal kinases responsible for phosphorylation and activation of the c-Jun and ATF2 transcription factors. The SAPK and the related p38 pathway are the first mammalian stress regulated pathways to be elucidated, although other pathways are being identified at a rapid pace (1,2,19). The SAPKs are a large ERK subgroup which is encoded on three genes each of which is subject to further diversification by three types of mRNA splicing into as many as 24 polypeptides (18,20). *In situ*, the SAPKs are activated by environmental, often genotoxic stresses (ionizing radiation, oxidative and chemical stress, heat shock, ischemic reperfusion injury) and by inflammatory cytokines (TNF and IL-1) (18). This activation is catalyzed in the cell by up to four chromatographically distinct MEKs, one of which SAPK/ERK kinase-1 (SEK1) has been cloned (21,22). MEKKs 1, 2 and 3, mammalian homologues of *S. cerevisiae STE11*, can, in turn, phosphorylate and activate SEK1 *in vitro* and *in situ* (6,7). MEKKs 2 and 3 can also activate the mitogenic pathway through activation of the p42/p44 MAPK activating MEK, MEK1 (7). In addition, SH3-domain-containing, proline-rich kinase (SPRK), a mammalian MLK which contains an SH3 domain as well as a CRIB domain (1,8), as well as Tpl-2 (9), the rat homologue of the product of the proto oncogene *cot* have been identified as additional kinases capable of activation of the SAPK pathways via direct SEK1 phosphorylation. The mechanisms of regulation of MEKKs, SPRK and Tpl-2 are unknown, however additional kinases (PAKs 1 and 3), as well as the mammalian *SPS1* homologue germinal center kinase (GCK) have been shown to be capable of activating the SAPK pathway in cotransfection experiments (10-12). Finally, both Rac1 and Cdc42Hs, which bind to both the PAKs and to SPRK, and which activate the PAKs, have been shown to lie upstream of the SAPKs (15,16).

The p38 pathway, although activated by essentially the same external stimuli as those activating the SAPKs, is regulated by largely distinct biochemical mechanisms (1,2). p38 is most closely related to the *S. cerevisiae* osmosensing ERK *HOG1* (1-3). Like the SAPKs, p38 phosphorylates and activates transcription factors (ATF2, Max, GADD153/CHOP) (1,2,23). In addition, p38 can activate other protein kinases (MAPK-activated protein kinase [MAPKAP] kinase-2) (1,2). MAPK kinase (MKK)3 and MKK6 are two novel MEKs which activate p38 specifically, without activation of SAPKs (24,25). SEK1 can also activate p38 (24); however, cotransfection experiments indicate that MEKK1, a candidate upstream activator of SEK1, cannot activate p38 (1,2). Whether this phenomenon represents molecular sequestration, or the existence of distinct SAPK-kinases which are MEKK1 substrates is unclear. To date only one MAPKKK has been identified for the p38 pathway. TGF-β-activated kinase-1 (TAK1), a novel member of the MEKK family, can activate MKK6 *in vitro*, and is activated itself, *in situ* by TGF-β (26). Rac1 and Cdc42Hs, likely through activation of the PAKs, can, in addition to activation of the SAPKs, activate the p38 pathway in cotransfection experiments (10,11). GCK, however, is incapable of p38 activation *in situ* unless massively overexpressed.

1.2 Subject, Purpose of Research

Our interest is to identify and elucidate the biochemistry and cell biology of the SAPK and p38 pathways, signal transduction pathways activated by stress and inflammatory cytokines, and then to characterize these pathways in breast cancer. Our ongoing experiments indicate that these pathways may inhibit cell growth and could thereby counteract the transformation process. In addition, these pathways may actually promote apoptosis (27,28). Once these pathways are understood, therefore, manipulation of stress signaling at the bedside, through novel therapeutic techniques, could prove efficacious in (1) limiting tumor growth through activation of stress signaling, or, conversely, (2) limiting the toxicity of genotoxic cancer chemotherapeutics by selectively inhibiting their activation of stress pathways.

1.3 Scope of Research (8/95-8/96)

1.3.1 *Biochemical Studies*

We sought during this year to identify and characterize additional biochemical components which regulate of the SAPK and p38 pathways. Specifically, the following studies were performed to these ends.

1) GCK was demonstrated to be a potent *in situ* activator of the SAPK pathway. This work has now been published (Pombo, C.M., *et al.* [1996] *Nature* **377**, 750-754)

2) We generated a series of recombinant GCK constructs wherein the C-terminal regulatory domain was progressively deleted. The effects of these deletions on SAPK activation and the specificity of GCK for the SAPKs was determined. These studies indicate that the C-terminal PEST domain (29) is necessary for optimal SAPK activation *in situ*, however all of the constructs retain their specificity for the SAPK pathway, suggesting that this specificity resides with the GCK catalytic domain.

3) We and others (30) have found that GCK binds to the small Ras superfamily GTPase Rab8. This interaction, however, does not result in SAPK activation, nor does it affect GCK activity. We are currently testing the possibility that Rab8 may affect GCK subcellular localization.

4) We have found that a novel MLK, receptor interacting protein (RIP), which was cloned based on its ability to interact with Fas and with proximal elements of TNF signaling (TNF receptor-associated death domain [TRADD])(31,32) can activate both the SAPK and p38 pathways. Biochemical assays indicate that RIP can directly activate MKK6, but cannot activate SEK1. We are currently completing these studies.

5) We have observed that the mammalian *STE20* homologue PAK1 can interact with SPRK, an MLK capable of activating SEK1 (8). This interaction occurs at the PAK C-terminal catalytic domain. We are mapping the binding sites on SPRK.

1.3.2 *Cell Cycle Studies*

We have developed a quantitative microinjection system useful for the study of the biological effects of expression of specific elements in the SAPK and p38 signaling pathways. These studies have yielded the following results.

1) Microinjection of p38 inhibits NIH3T3 cell cycle progression at the G_1/S transition point. SAPK or GCK microinjection are essentially without effect on cell cycle progression.

2) Injection of SEK1, MKK3 or MKK6 blocks G_1/S transition, suggesting a requirement for active p38. Consistent with this, coinjection of p38 and kinase-dead, dominant inhibitory SEK1 or MKK3 constructs blocks the ability of p38 to arrest cells at G_1/S.

3) Injection of Cdc42Hs into cells also blocks G_1/S progression by a mechanism which requires active p38 and which can be partially reversed with kinase dead MKK3 and SEK1. Rac1, which can also activate p38 and the SAPKs in cotransfection experiments (15), fails to arrest cells in G_1.

Having developed this system and proven in simple cell models its overall feasibility, we can now adapt it for the study of more complicated cell types, including breast cancer cells.

2. BODY

2.1 Biochemical Studies

These studies were intended to identify and characterize molecular components upstream of the SAPKs and p38. Our principal focus has been on GCK, a mammalian homologue of *S. cerevisiae SPS1*. We have also performed studies on RIP, SPRK and PAK1. These experiments are now described.

2.1.1 *Studies of GCK*

1) *GCK deletion series*. GCK contains an N-terminal Ser/Thr catalytic domain followed by an extensive C-terminal regulatory domain which is comprised of three PEST sequences and a leucine rich region near the extreme C-terminus (29). Previous studies from our laboratory indicated that expression of GCK resulted in activation of the SAPK pathway in the absence of external stimuli, suggesting that GCK was constitutively active and regulated by limiting concentrations of an inhibitor, or by oligomerization (12). Our initial studies indicated that this regulation was likely mediated by the GCK C-terminus (12). Our results also indicated that expression of GCK did not activate the p38 or MAPK pathways (12). To characterize the molecular bases for these properties, we constructed a GCK deletion series in order to map GCK regulation and pathway specificity. Fig. 1 illustrates schematically these constructs.

Deletion series for GCK analysis of SAPK pathway activation

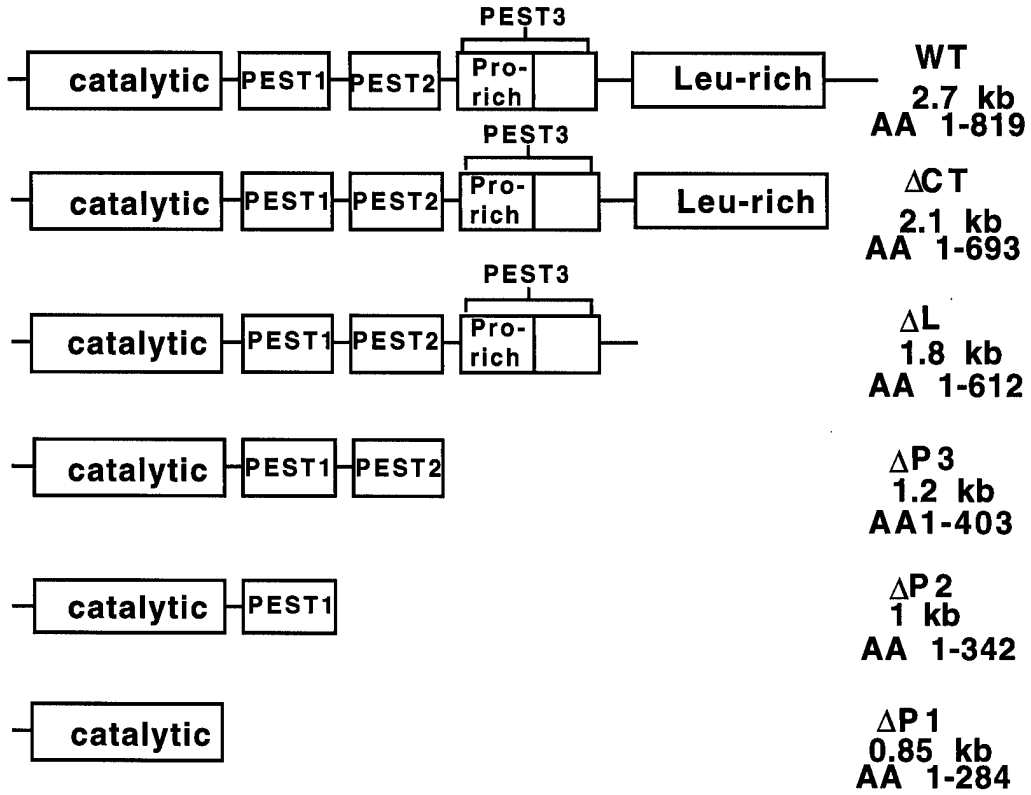

Fig. 1. Schematic of GCK deletion series. Constructs were generated by PCR and cloned into the mammalian expression vector pCMV5-M2-FLAG. Accordingly, constructs were expressed with an M2-FLAG epitope for monitoring expression levels.

We transfected transiently very small amounts (0.3 μg per 10 cm dish) of the GCK deletion series into 293 cells along with HA-tagged SAPK and GST-tagged p38. Activation of SAPK was measured using HA immunoprecipitates with GST-c-Jun as a substrate while p38 was measured using ATF2 as a substrate after recovery on glutathione agarose. GCK activity was assessed using MBP as a substrate. Fig. 2 shows the results of these studies.

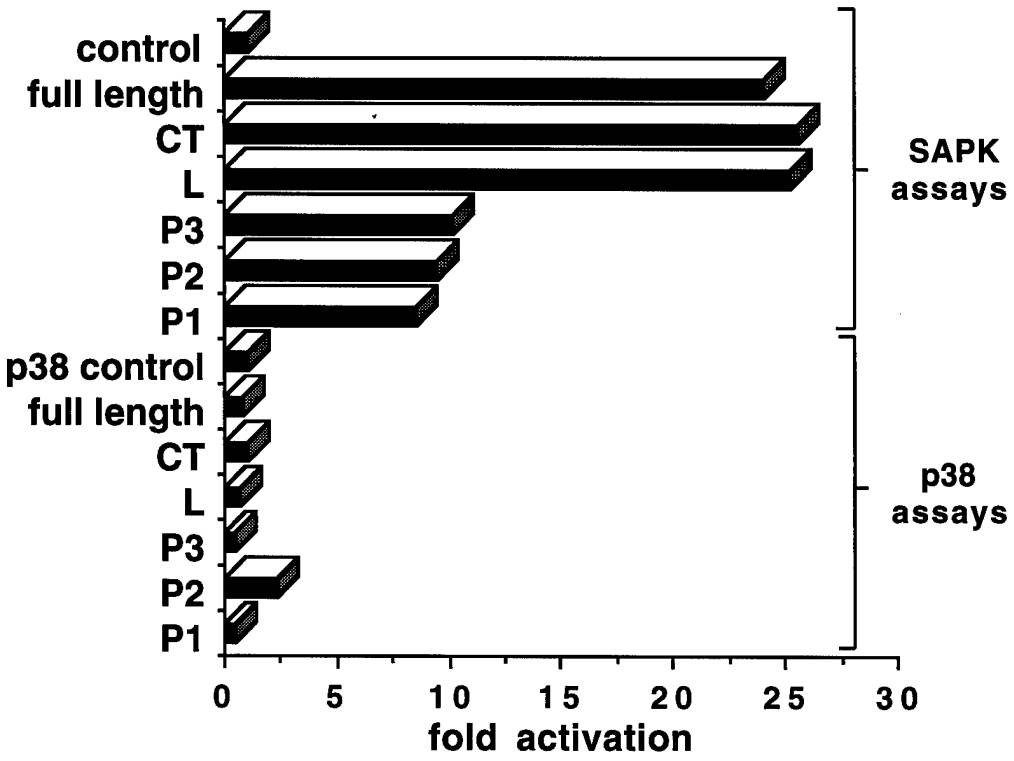

Fig. 2. Cotransfection of GCK deletion series with SAPK and p38: effects on SAPK and p38 activity. 293 cells were transfected with a small amount of FLAG-GCK constructs (0.3 μg) along with HA-SAPK (1 μg) or GST-p38 (1 μg). Expression of the GCK constructs was equal in all transfections as judged by western blotting. Activity of the GCK constructs was also measured and was essentially equal for all transfections. SAPK and p38 activities are shown.

It is clear that while all of the deletion constructs can substantially activate the SAPK pathway, deletion of the C-terminal PEST domain abrogates significantly SAPK activation. This suggests that the C-terminal PEST motif contains elements necessary for optimal activation of the SAPK pathway. By contrast, all of the deletion constructs retain their specificity for the SAPK pathway, and do not activate the p38 pathway. If much larger amounts of GCK are transfected (5 μg), some modest p38 activation is seen for each of the deletion constructs. However, even upon expression of large amounts of GCK, no activation of the mitogenic ERKs is observed for any of the GCK deletions. We conclude that specificity of the GCK for the SAPK pathway resides principally in the GCK catalytic domain.

2) *The small GTP binding protein Rab8 interacts with GCK.* A recent report in the literature (30) described a yeast two hybrid screen, using Rab8 as bait, which identified GCK as a potential Rab8 interactor. Rab8 is a member of the ARF subgroup of the Ras superfamily. Up until now, Rab8 had not been implicated in regulation of protein kinase signaling. Instead, Rab8 is involved in the regulation of membrane vesicle trafficking between the *trans* Golgi and the plasma membrane (33,34). Our two hybrid screens using GCK as bait did not reveal Rab8 as a binding protein for GCK. Accordingly, we sought to explore this phenomenon biochemically. We cloned wild type Rab8 by PCR, and Johan Perainen of the University of Helsinki was kind enough to provide mutant Rab8 constructs--22N, a GTPase-deficient,

constitutively active Rab8 and 67L, a dominant inhibitory mutant (33,34). Using HA-tagged, wild type Rab8 and FLAG-tagged GCK constructs in cotransfection experiments, we sought to determine if Rab8 could indeed interact with GCK *in situ*, and could be recovered in GCK immunoprecipitates. From Fig. 3, it is clear that Rab8 can bind GCK and that this interaction requires the extreme C-terminal 126 amino acids of GCK, inasmuch as only wild type GCK could bind Rab8.

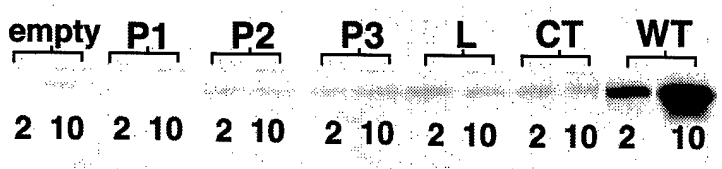

Fig. 3. Co-immunoprecipitation of Rab8 and GCK deletion constructs. 293 cells were cotransfected with HA-tagged wild-type Rab8 (μg Rab8 plasmid transfected are listed below each lane) and 5 μg of the M2-FLAG-tagged GCK deletion constructs indicated above each lane (Empty denotes empty pCMV5-FLAG vector. See Fig. 1 for definition of deletion constructs). GCK Rab8 complexes were recovered by immunoprecipitation with anti M2-FLAG and immunoblotted with anti HA antibody to detect bound Rab8.

Insofar as only wild type Rab8 could bind GCK, while all of the deletion constructs could activate SAPK substantially (See Fig. 2), we surmised that Rab8 likely did not regulate the ability of GCK to activate the SAPKs. To confirm this, we tested whether or not (1) a constitutively active, GTPase-deficient Rab8 mutant (22L) could activate SAPK in a manner analogous to that engendered by constitutively active (V12) Cdc42Hs; and (2) if a dominant inhibitory Rab8 mutant deficient in GDP/GTP exchange (67L) could abrogate GCK activation of SAPK. From Fig. 4, it is clear that these Rab8 mutants had no effect on SAPK activation. We conclude that the interaction of GCK and Rab8 serves a purpose distinct from activation/regulation of the SAPK pathway. Given that Rab8 has been clearly shown to influence vesicular trafficking (33,34), it is possible that Rab8 serves to bring the GCK → SAPK machinery to specific substrates in Rab8 target vesicles. We are currently analyzing this possibility.

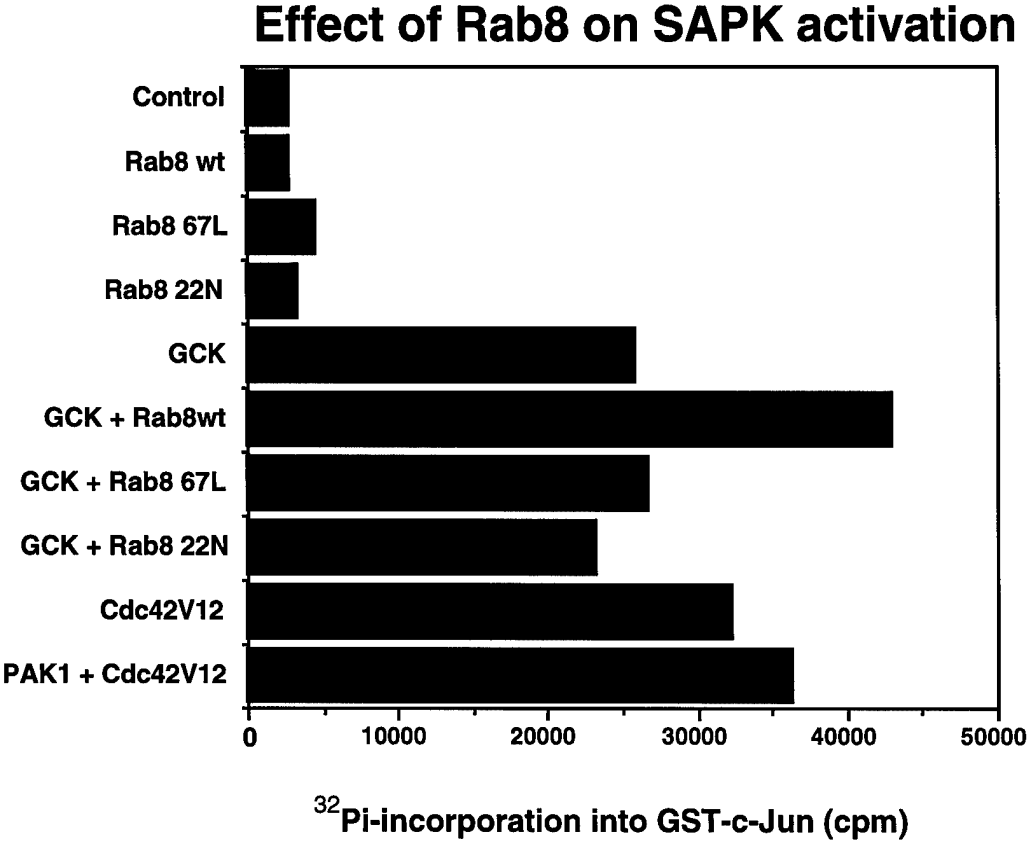

Fig. 4. Effect of Rab8 on SAPK activation and GCK regulation of SAPKs. 293 cells were cotransfected with HA-SAPK (1 μg), HA-tagged Rab8 constructs (10 μg), wild type M2-FLAG GCK (0.3 μg), M2-FLAG V12Cdc42Hs and M2-FLAG-PAK1 (10 μg each) as indicated. SAPK was immunoprecipitated with anti HA antibody and assayed for phosphorylation of GST-c-Jun. Differences in SAPK activity in the GCK cotransfected assays reflect minor differences in GCK expression as judged by immunoblot.

2.1.2 *RIP activates the SAPK and p38 pathways: Direct regulation of MKK6 but not SEK1*

1) *Activation of the SAPK and p38 pathways by RIP*. RIP is a novel protein kinase similar in its catalytic domain to the MLKs (1,31). It was originally cloned based on its ability to interact with Fas, a transmembrane receptor of the TNF family (31,35). Both Fas and the RIP C-terminal region contain a so-called death domain. This is a stretch of amino acids which is required for the recruitment and activation of apoptotic machinery (35). Not surprisingly, Fas recruitment of RIP or RIP overexpression can cause apoptosis (31). RIP can also interact indirectly with the TNF receptor machinery. The 55-kDa TNF receptor binds a 30-kDa polypeptide, TNF receptor-associated death domain (TRADD) in a TNF-dependent manner (36). TRADD can also recruit other adapter molecules to the TNFR (37,38). Recent studies have demonstrated that TRADD also binds RIP (32). Accordingly, TNF-dependent recruitment of TRADD to the TNF receptor can bring TRADD-RIP to the membrane. This may serve to regulate RIP.

Inasmuch as TNF, possibly acting through RIP, is a potent activator of both the SAPKs and p38 (1,2,18), we sought to determine if RIP overexpression could activate the SAPK and p38 pathways. We obtained Myc-tagged RIP from Brian Seed and employed this construct in cotransfection experiments which clearly demonstrate that RIP overexpression activates both the SAPK and p38 pathways (Fig. 5).

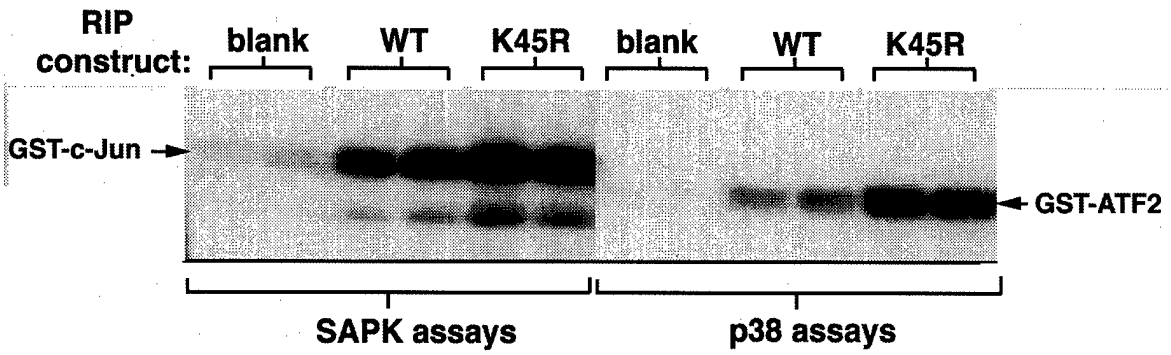

Fig. 5. Activation of SAPK and p38 upon coexpression with RIP or kinase-dead (K45R) RIP. 293 cells were cotransfected with HA-tagged SAPK, GST-tagged p38 and either wild type (WT) RIP or a kinase-dead RIP mutant (K45R) wherein the lysine critical for ATP binding was mutated to arginine. SAPK and p38 were recovered from cell extracts by anti HA immunoprecipitation or glutathione agarose chromatography, respectively, and assayed for c-Jun kinase (SAPK) or ATF2 kinase (p38). Blank cotransfections employed the empty cognate vector carrying the RIP plasmids.

It is noteworthy that the kinase-dead (K45R) RIP is apparently equipotent, if not more potent at SAPK and p38 activation than is wild type RIP. This is consistent with reports indicating that the kinase-dead RIP could also stimulate apoptosis (31). The degree of activation by the K45R construct is deceptive, however, as this construct is expressed much more highly than the wild type (See Fig. 6). Still, these results imply that RIP must not only be able to activate the SAPKs and p38 directly, but it must also be able to recruit parallel SAPK and p38 activation machinery.

2) *RIP is a direct activator of MKK6.* We have shown that SPRK, an MLK, can directly activate the SAPK activating MEK, SEK1 (1,8). Inasmuch as RIP bears some similarity to the MLKs (1,31), we sought to determine if RIP were able to activate directly MEKs upstream of the SAPKs and p38. To do this, wild type and kinase-dead RIP were immunoprecipitated from transfected cells and assayed *in vitro* for activation of MKK6 or SEK1 using a robotic assay wherein the activated MEK is then assayed for activation of the cognate ERK (4). From Fig. 6, it is clear that whereas RIP can directly activate MKK6, it cannot activate SEK1 under conditions in which a bacterially expressed MEKK1 can.

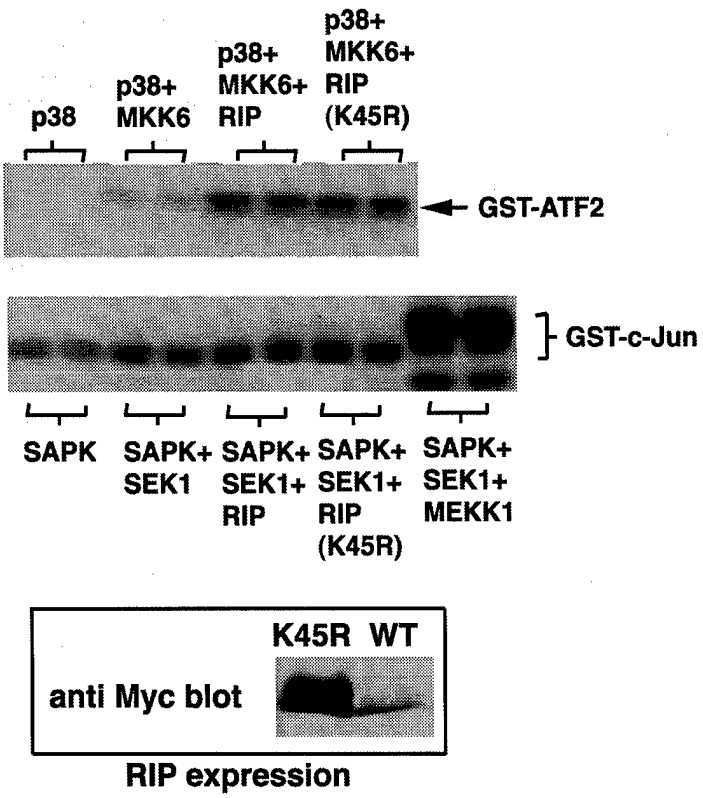

Fig. 6. Direct activation of MKK6, but not SEK1 by RIP. Myc-tagged RIP or K45R kinase-dead RIP were transfected into 293 cells and the expressed protein immunoprecipitated with the anti Myc antibody. The immunoprecipitates were assayed for activation of GST-tagged MKK6 or SEK1. As a positive control, MEKK1 expressed and purified from bacteria was used to activate SEK1. Expression of the two RIP constructs is shown in the boxed insert. Control assays eliminating MKK6/SEK and/or p38/SAPK from the assays including RIP yielded no detectable activity (not shown).

As was observed for the *in situ* activation of SAPK and p38 (Fig. 5), the KR-RIP can activate MKK6. However, as can be seen in Fig. 6, equal MKK6 activation is seen under conditions wherein there is at least 10 fold more KR-RIP expressed than WT-RIP. We conclude from this that either the catalytic activity of RIP directly activates MKK6, while the noncatalytic domain associates with additional MKK6 activating kinases, or RIP catalytic activity is necessary for association of MKK6 kinases. As for the lack of apparent SEK1 activation, at least four chromatographically distinct SAPK-activating MEKs have been detected in 3Y1 cells (22). It is therefore likely that RIP activates SAPKs through a MEK distinct from SEK1.

2.1.3 *Detection of an interaction between PAK1 and SPRK and homodimerization of PAK1*

SPRK is an SH3 domain- and CRIB domain-containing MLK which can directly phosphorylate and activate SEK1 (1,8,16). PAK1 is a mammalian *STE20* homologue which activates SAPK or p38 upon coexpression and is itself activated upon binding Cdc42Hs or Rac1, both of which have been implicated as upstream activators of the SAPKs and p38 (1,2,10,11,15,39). Genetic epistasis studies suggest that *STE20*s and kinases like them are upstream of MAPKKKs (3). Moreover, PAK1 has several consensus SH3 binding sites in its amino terminal regulatory domain (1,2). Accordingly, we sought to determine if these could bind SPRK. In cells coexpressing M2-FLAG SPRK and GST-PAK, we were able to detect GST-PAK in SPRK immunoprecipitates, indicating an interaction between these two regulators of stress signaling (Fig. 7, lane1). To our surprise, however, the PAK N-terminus (AAs 1-232, containing the

consensus SH3 binding sites) did not bind SPRK, and the PAK-SPRK interaction required the PAK C-terminal 322 amino acids (Fig. 7, lanes 2 and 3).

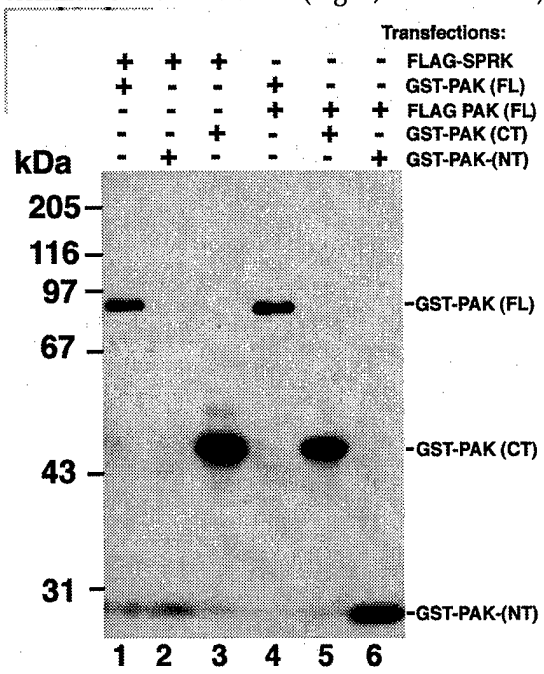

Fig. 7. Detection of an interaction between PAK1 and SPRK. 293 cells were cotransfected with GST full length, amino terminus (NT, amino acids 1-232), carboxyterminus (CT, amino acids 232-544), M2-FLAG-tagged full length PAK1 or SPRK as indicated. M2-FLAG-tagged proteins were recovered by immunoprecipitation and bound GST-tagged polypeptides detected by immunoblotting with anti-GST antibody.

In addition to detecting a heterologous interaction between SPRK and PAK, we were also able to detect PAK homodimers. (Fig. 7, lanes 3-6). In this case, PAK1 could interact with either the N-terminus or the C terminal domain (missing the SH3 binding sites and the CRIB domain). The significance of the PAK-SPRK and PAK-PAK interactions is unclear. Recently, the Raf-1 MAPKKK was shown to be activated in part upon homo-oligomerization (40). Perhaps a similar mechanism is involved in PAK regulation. Once activated PAKs may activate SPRK. Alternatively, inasmuch as SPRK, like PAK contains a CRIB domain, but cannot, as PAK can, activate p38, it is possible that the PAK-SPRK interaction allows for Rho family GTPases to regulate common and distinct pathways downstream of PAK and SPRK.

2.2 Regulation of Cell Cycle Progression by the Cdc42Hs → p38 pathway

In addition to characterizing the biochemistry of stress signaling, we sought to understand the biological functions of the various signaling components which we had identified. To this end we wished to develop a quantifiable bioassay system which could accurately reflect the biology of SAPK and p38 signaling elements. Microinjection proved the most ideal for this sort of study. High levels and efficiencies of expression were possible and the expressing cells could easily be counted for particular biological functions. Our initial studies employed a simple system, the cell division cycle of the NIH3T3 cell, which can easily be arrested in G_0 by serum withdrawal. Cell cycle progression in these cells is also easily assayed using a variety of techniques. Our results, however, were quite unexpected, and point to the complexity of function of the SAPK and p38 pathways.

2.2.1 *Microinjection of p38, but Not SAPK inhibits NIH3T3 cell cycle progression at G_1/S*

Previous studies have indicated that the Ras-regulated MAPK cascade is required for cell growth (17). Moreover, many of the stimuli which activate the SAPK and p38 pathways (e.g., UV and γ radiation,

chemical DNA damage) arrest the cell cycle at G_1 in order to allow for DNA and cellular repair (1,2). Such cell cycle checkpoints are essential to cellular survival and to the prevention of cellular transformation in response to genotoxic agents. Accordingly, we wished to test directly if either the SAPK or p38 pathways could affect cell cycle progression. NIH3T3 cells were serum starved in order to arrest and synchronize the cell cycle at G_0. Cells were then plated onto coverslips in 10% serum in order to re-initiate synchronously the cell cycle and were then microinjected with expression plasmids encoding various signaling proteins. Cells expressing recombinant proteins (stained for FLAG or HA tags) were scored for G_1/S transition by staining for BrDU incorporation.

We microinjected plasmids encoding p38, SAPK (p46-β1), p44 MAPK and p70 S6 kinase and observed that p38 expressed from microinjected plasmid markedly arrested cell cycle progression at G_1/S (Fig. 8).

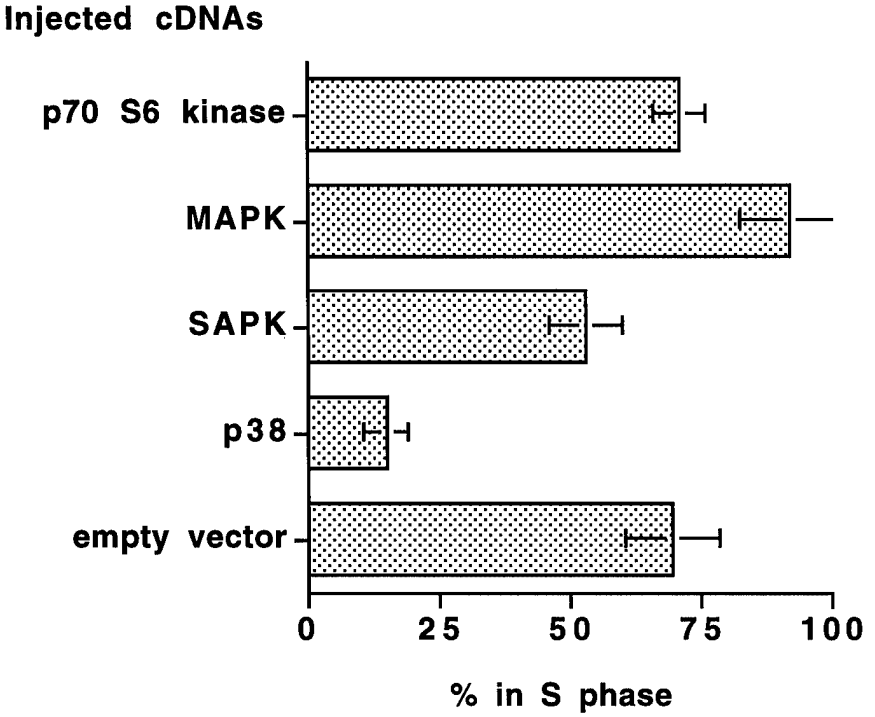

Fig. 8. Expression of microinjected p38 inhibits G_1/S transition in NIH3T3 cells. Cells were synchronized and microinjected as described above. S phase entry was measured as described above.

Surprisingly, SAPK, which is activated by the same antimitogenic stimuli as p38 (1,2,18) did not significantly arrest cells in G_1. Staining of microinjected cells for active p38 with an antibody against the phosphorylated, active form of p38 (1,2) revealed that microinjection increased the level of active p38 in the cells. Similarly, cells injected with SAPK, in spite of showing no growth inhibition, contained elevated levels of active SAPK as assessed by staining with an antibody specific for c-Jun phosphorylated by SAPK (1,2). To confirm further that SAPK did not arrest cells in G_1, we microinjected cells with GCK, a specific SAPK activator (Fig. 2 and ref. 12). As can be seen in Fig. 9, GCK does arrest cells in G_1. However, this arrest is likely due to the promiscuous activation of p38 which can occur upon massive GCK overexpression. In support of this, coinjection of GCK with a kinase-dead, dominant inhibitory MKK3 construct completely blocks GCK inhibition of G_1/S transition, even if SAPK is coinjected.

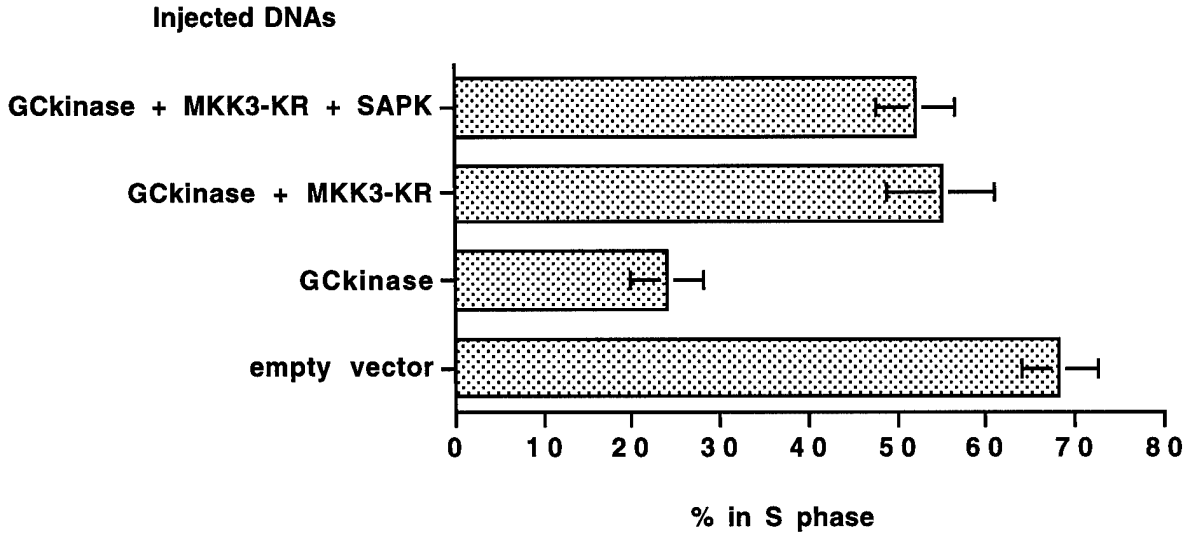

Fig. 9. SAPK is likely not able to arrest cells in G_1. Inhibition of G_1/S transition by GCK is due to nonspecific activation of p38. Microinjection experiments were performed as described. GCK was FLAG-tagged, SAPK and MKK3-KR were HA tagged.

2.2.2 *MEKs upstream of p38 inhibit G_1/S progression*

To confirm that activation of p38 was a necessary step in G_1/S arrest observed upon p38 microinjection, we tested three of the MEKs known to lie upstream of p38: SEK1, MKK3 and MKK6 (21,24,25). All three of these MEKs were able to potently arrest cells at the G_1 restriction point. Insofar as kinase-dead mutants of MKK3 or SEK were unable to arrest the cell cycle, it is clear that active MEKs and, therefore active p38 are necessary for G_1 arrest (Fig. 10).

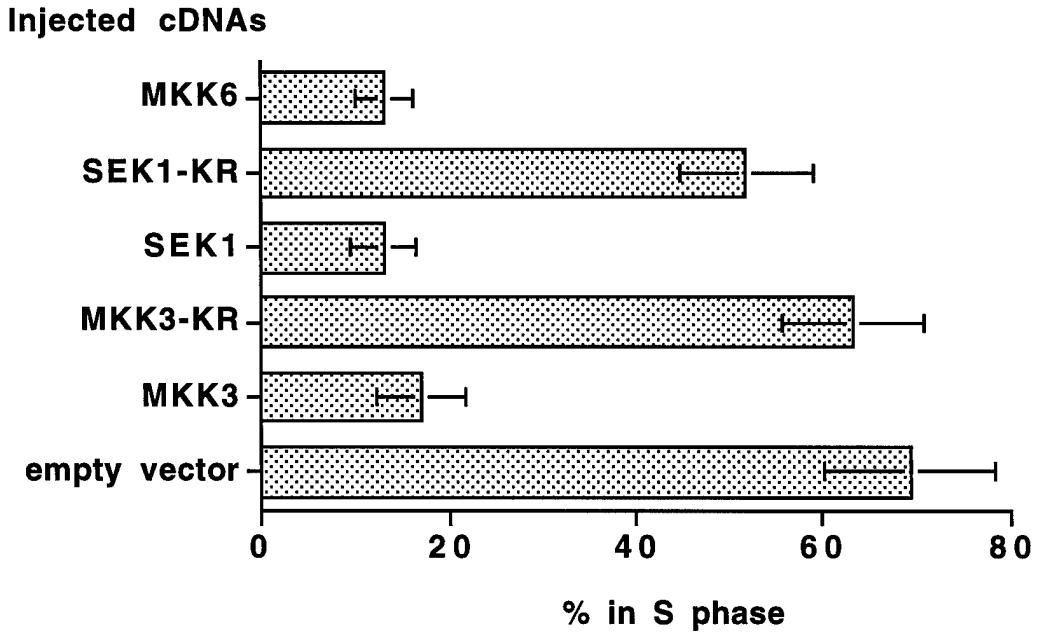

Fig. 10. MEKs upstream of p38 arrest cells in G_1. inactive MEKs do not arrest the cell cycle. Cells were injected with the constructs indicated (all HA-tagged, except MKK6 which was FLAG tagged. G_1 arrest was assessed as described above.

Further confirmation of the requirement for active p38 in cell cycle arrest came from the use of kinase-dead MEKs as inhibitors of activation of coinjected p38 (24,25). In the experiment shown in Fig. 11, cells were microinjected with either p38 alone or p38 plus KR-MKK3 or SEK1. The kinase-dead MEKs were able to inhibit p38-mediated G_1 arrest completely.

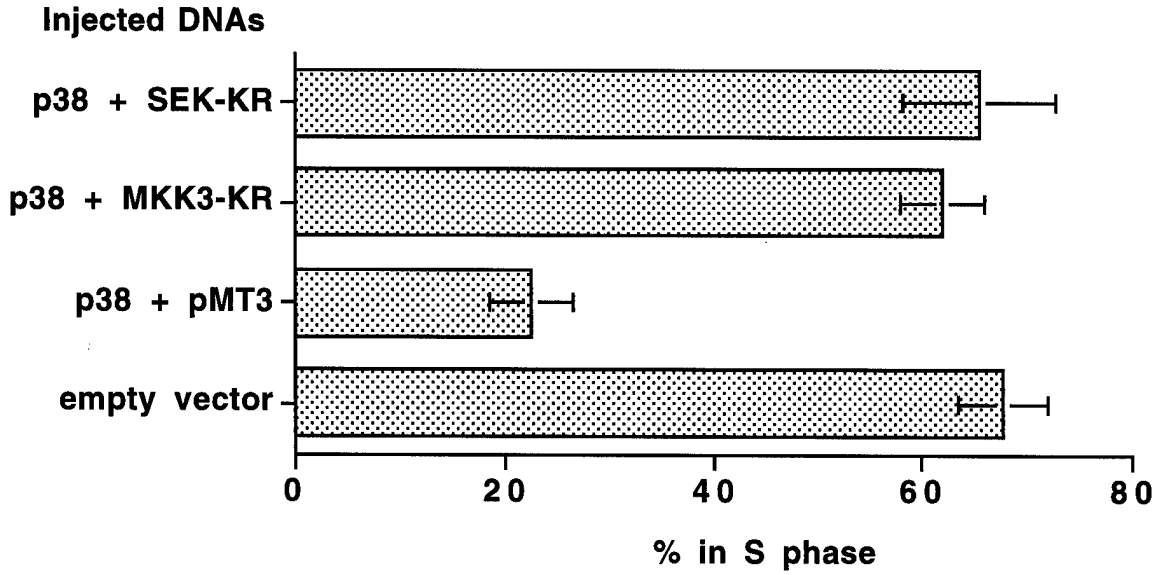

Fig. 11. Kinase-dead, dominant inhibitory mutants of MKK3 or SEK1 can inhibit p38-mediated G_1 arrest. All injected constructs were HA-tagged. pMT3 is the empty parent vector used in this experiment. Staining with an antibody to p38 indicated that p38 was still overexpressed in the KR-MKK3 and KR-SEK-injected cells.

2.2.3 *The Rho family GTPase Cdc42Hs inhibits G_1/S transition in part by activation of p38*

Both p38 and the SAPKs can be activated *in situ* by Cdc42Hs and Rac1, two members of the Rho subgroup of the Ras superfamily (10,11,15). To test if either of these small GTPases could arrest the cell cycle, we microinjected expression plasmids encoding Cdc42Hs, Rac and, as a negative control, RhoA. As can be seen in Fig. 12, wild type Cdc42Hs strikingly inhibits cell cycle progression to S phase. By contrast, neither wild type nor a constitutively active allele of Rac1 (V12) markedly inhibits G_1/S transition. Similarly, V12-RhoA, which does not activate SAPK or p38, does not block S phase entry. Moreover, the inhibition of cell cycle progression mediated by Cdc42Hs likely requires active p38 inasmuch as kinase-dead, dominant inhibitory MKK3 and SEK1 coinjected with Cdc42Hs block the ability of Cdc42Hs to arrest the cell cycle at G_1.

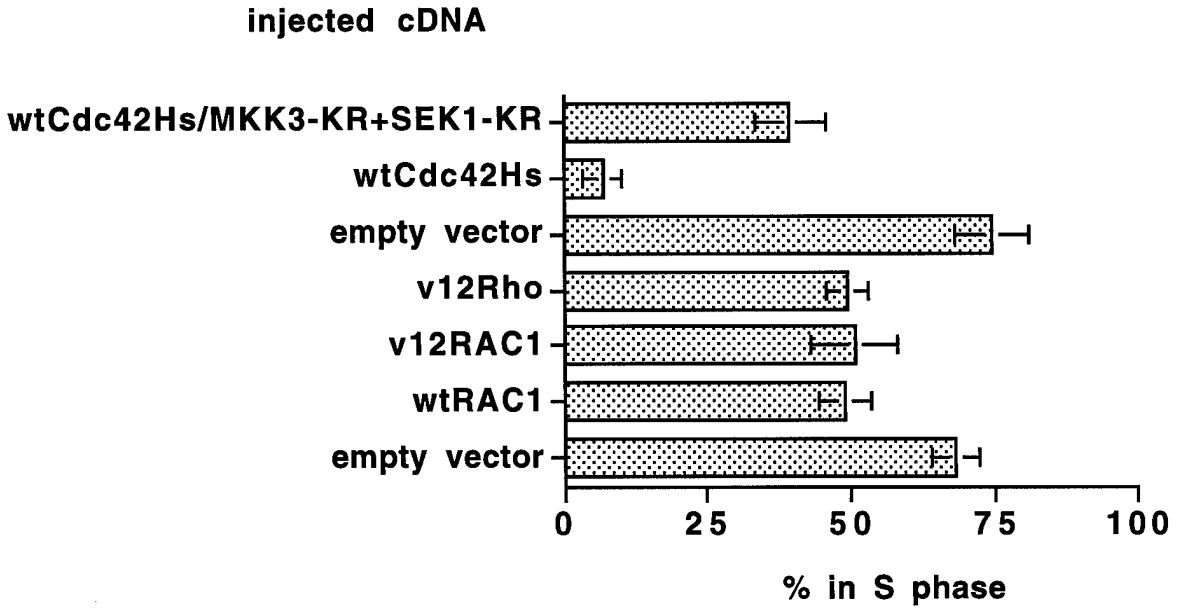

Fig. 12. Inhibition of G_1 progression mediated by Cdc42Hs by a p38-dependent mechanism. All constructs injected were M2-FLAG-tagged except the KR-MEKs which were HA-tagged. Empty vector for the upper bar was a combination of the FLAG and HA vectors (pCMV5 and pMT3). For the lower bar, the empty plasmid injected was pCMV5. Activity of the Rac construct was confirmed by staining for membrane ruffling, a classical cellular response to Rac1 activation (1,2). RhoA activity was monitored by staining for actin stress fibers in the injected cells (1,2).

3. CONCLUSIONS

Our studies have been divided into biochemical characterizations of the mechanisms of SAPK and p38 regulation, and cell biological characterizations of the effects of these pathways on cell growth.

3.1 Biochemical Studies

Fig. 13 is a model which summarizes our conclusions concerning the biochemistry of SAPK and p38 regulation.

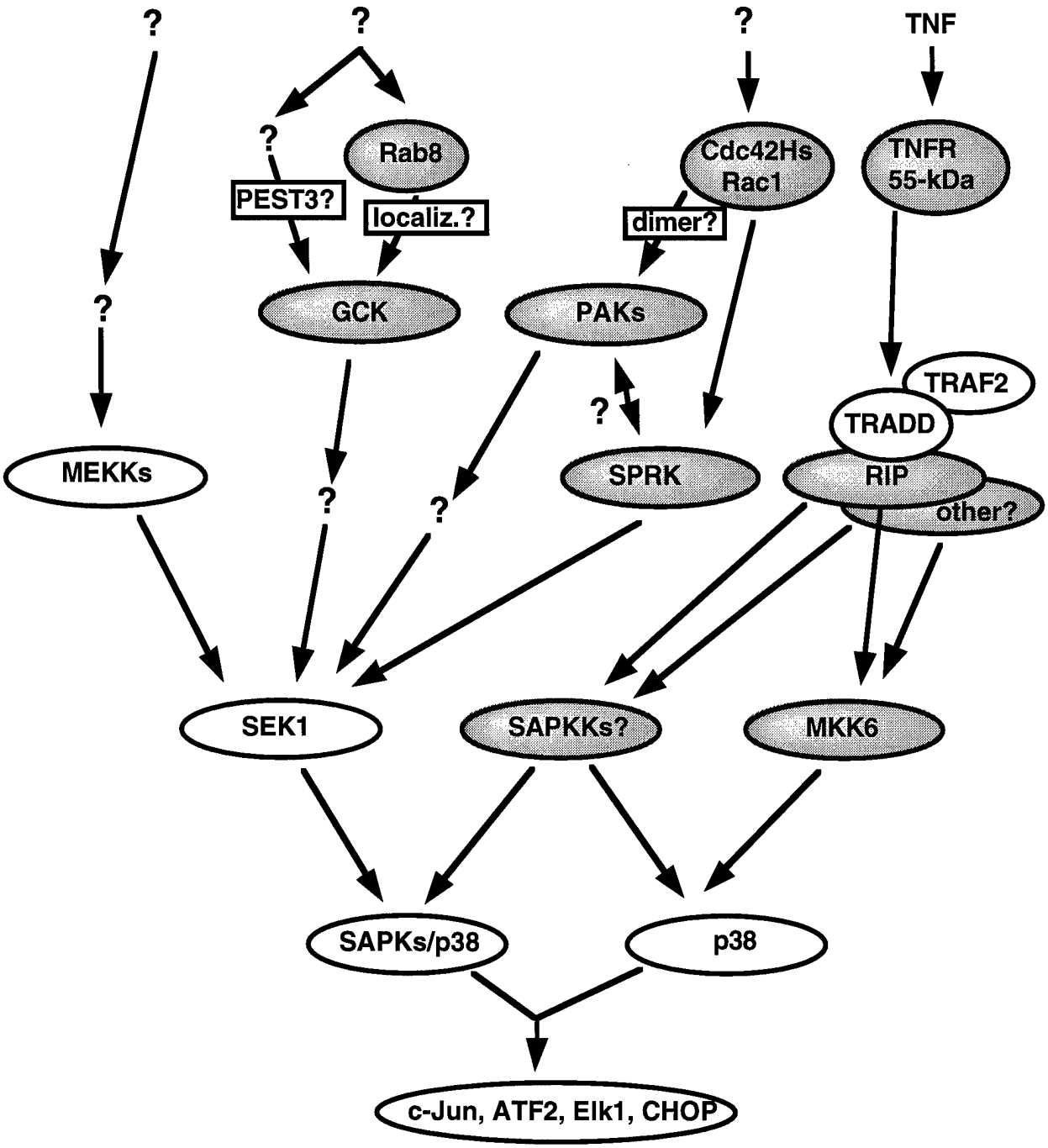

Fig. 13. Model of the biochemical steps in stress signal transduction. Highlighted in gray are the specific areas addressed in the funding period of this report.

Several things become clear from this model. First, we have identified a great many upstream activators for the SAPKs and p38 many of which have not been tied to extracellular signals. For example, what ligands activate MEKKs?, SPRK?, GCK?, PAK1?, Cdc42Hs? Second, the molecular connections between *STE20* and *SPS1* homologues and their downstream targets remain obscure. Although we can detect a molecular interaction between SPRK and PAK1, the physiologic significance of this interaction remains to be determined. With the exception of RIP, the upstream regulation of the MAPKKKs upstream of the SAPKs and p38 is also unknown. Finally, which of these diverse signals is expressed in

breast cancer cells and what is the biology of these proteins in breast cancer. Our efforts at cloning, expression and assay of these SAPK and p38 regulators has now begun to yield results. We have initially used model cell systems for our biochemical studies in deference to the ease of analysis using these cells. With the information now generated, we will begin to pursue biochemical studies in breast cancer cells.

3.2 Cell Biological Studies

Fig. 14 is a model which summarizes our results from the microinjection experiments.

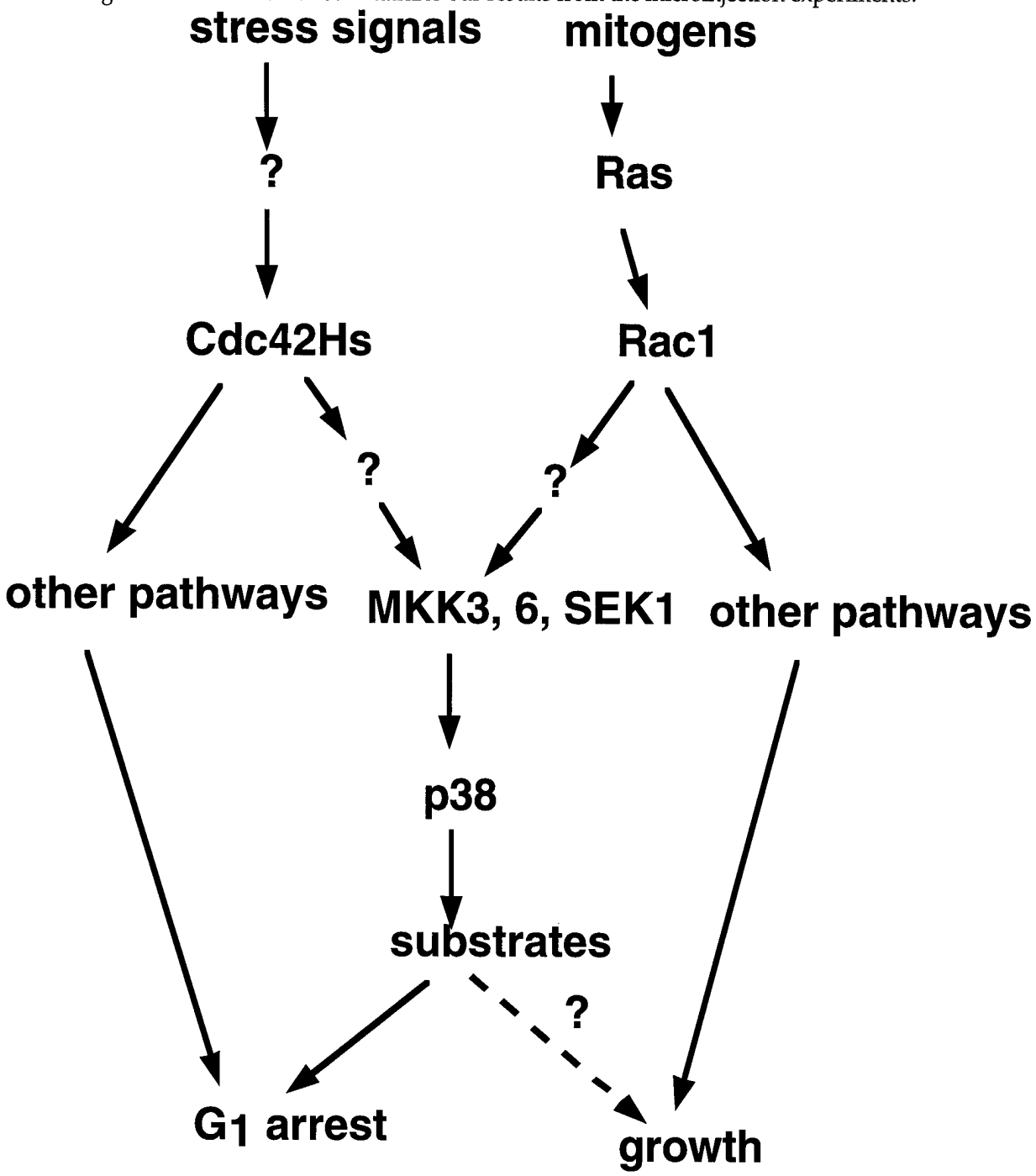

Fig. 14. Model of the effects of stress signaling on cell cycle.

We propose that activation of Cdc42Hs is antimitogenic, whereas activation of Rac1 is not and may, as has been indicated in the literature (1,2), be mitogenic. Both pathways, however, activate the SAPKs and p38. Activation of p38 appears to be obligatory for cell cycle arrest in response to activation of Cdc42Hs. However, it is likely that Cdc42Hs and Rac exert their effects on multiple pathways and the opposing effects of Cdc42 and Rac1 activation may involve these other pathways and may also require p38. What this means is that therapies which address p38 activation, including drugs which arise from the novel pyridinyl imidazole p38 inhibitors (41), may not necessarily prevent or promote breast cancer cell growth and care must be taken to assess carefully the roles of these stress pathways in cell growth. In addition, we do not know what the effects on the cell cycle are of activation of multiple Ras family signaling pathways. Indeed, Cdc42Hs may exhibit different properties when activated in conjunction with parallel pathways. Most importantly, the techniques and results summarized in Fig. 14 make possible the study of the role of these pathways in the growth of transformed breast cancer cells. Finally, the results in Figs. 13 and 14 are not mutually exclusive. As new molecular players in SAPK and p38 regulation are identified, they can be subjected to the biological studies already developed for the p38 NIH3T3 microinjection studies.

4. REFERENCES

1. Kyriakis, J.M. and Avruch, J. (1996) J. Biol. Chem. 271, 24313-24316.
2. Kyriakis, J.M. and Avruch, J. (1996) BioEssays 18, 567,577.
3. Herskowitz, I. (1995) Cell 80,187-197.
4. Kyriakis, J.M. et al. (1992) Nature 358, 417-421.
5. Lange-Carter C.A. et al. (1993) Science 260, 315-319.
6. Yan, M. et al. (1994) Nature 372, 798-800.
7. Blank, J.L. et al. (1996) J. Biol. Chem. 271, 5361-5368.
8. Rana, A. et al. (1996) J. Biol. Chem. 271, 19025-19028.
9. Salmerón, A. et al. (1996) EMBO J. 15, 817-826.
10. Zhang, S. et al. (1995) J. Biol. Chem. 270, 23934-23936.
11. Bagrodia, S., Dérijard, B., Davis, R.J. and Cerione, R.A. (1995) J. Biol. Chem. 270, 27995-27998.
12. Pombo, C.M. et al. (1995) Nature 377, 750-754.
13, Freisen, H., Lunz, R., Doyle, S. and Segall, J. (1994) Genes Dev. 8, 2162-2175.
14. Zhang, X.-f et al. (1993) Nature 364, 308-313.
15. Coso, O.A. et al. (1995) Cell 81, 1137-1146.
16. Burbelo, P.D., Drechel, D. and Hall, A. (1995) J. Biol. Chem. 270, 29071-29074.
17. Avruch, J., Zhang, X.-f. and Kyriakis, J.M. (1994) Trends Biochem. Sci. 19, 279-283.
18. Kyriakis, J.M. et al. (1994) Nature 369, 156-160.
19. Pombo, C.M. et al. (1996) EMBO J. 15, 4537-4546.
20. Gupta, S. et al. (1996) EMBO J. 15, 2760-2770.
21. Sánchez, I. et al. (1994) Nature 372, 798-800.
22. Moriguchi, T. et al. (1995) J. Biol. Chem. 270, 12969-12972.
23. Wang, X. and Ron, D. (1996) Science 272, 1347-1349.
24. Dérijard, B. et al. (1995) Science 267, 682-685.
25. Raingeaud, J. et al. (1996) Mol. Cell. Biol. 16, 1247-1255.
26. Moriguchi, T. et al. (1996) J. Biol. Chem. 271, 13675-13679.
27. Xia, Z. et al. (1996) Science 270, 1326-1331.
28. Verhiej, M. et al. (1996) Nature 380,75-79.
29. Katz, P., Whalen, G. and Kehrl, J.H. (1994) J. Biol. Chem. 269, 16802-16809.
30. Ren, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93, 5151-5155.
31. Stanger, B.Z. et al. (1995) Cell 81, 513-523.
32. Hsu, H. et al. (1996) Immunity 4, 398-396.
33. Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) Nature 348, 125-132.
34. Chavrier, P. et al. (1990) Mol. Cell. Biol. 10, 6578-6585.
35. Vandenabeele, P., Declercq, W., Beyaert, R. and Fiers, W. (1995) Trends Biochem. Sci. 5, 392-399.
36. Hsu, H., Xiong, J. and Goeddel, D.V. (1995) Cell 81, 495-504.

37. Hsu, H., Shu, H.-B., Pan, M.P. and Goeddel, D.V. (1996) Cell 84, 299-308.
38. Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994) Cell 78, 681-692.
39. Manser, E. et al. (1994) Nature 367, 40-46.
40. Luo, Z. et al. (1996) Nature 383,181-185.
41. Lee, J.C. et al. (1994) Nature 372, 739-746.

5. BIBLIOGRAPHY AND PERSONNEL

5.1 Bibliography

The following papers have been published, are in press or have been submitted during this funding period (8/85-8/96).

1. Pombo, C.M.,Kehrl, J.H., Sánchez, I., Katz, P., Avruch, J., Zon, L., Woodgett, J.R., Force, T. and Kyriakis, J.M. (1996) Activation of the SAPK pathway by the human *STE20* homologue germinal centre kinase. *Nature* **377,** 750-754.

2. Kyriakis, J.M. and Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays* **18,** 567-577.

3. Rana, A., Gallo, K., Godowski, P., Hirai, S.-i, Ohno, S., Zon, L., Kyriakis, J.M. and Avruch, J. (1996) The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator SEK1. *J. Biol. Chem.* **271,** 19025-19028.

4. Pombo, C.M., Bonventre, J.V., Molnár, Á., Kyriakis, J.M. and Force, T. (1996) Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway. *EMBO J.* **15,** 4537-4546.

5) Kyriakis, J.M., and Avruch, J. (1996) Sounding the alarm: Protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271,** 24313-24316.

6) Molnár, Á., Theodoras, A.M., Zon, L.I. and Kyriakis, J.M. (1996) Inhibition of cell cycle progression at G_1/S by Cdc42Hs activation of the p38/RK signaling pathway. (manuscript submitted).

5.2 Personnel

The following personnel were paid from this project during the funding period 8/95-8/96.

Irma Sánchez, Ph.D. (until 9/30/95)
Árpád Molnár, M.D.
Anthony Makkinje, Ph.D. (until 7/1/96)
John M. Kyriakis, Ph.D.