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13. ABSTRACT (*Maximum 200 words*) We have characterized a mammalian signal transduction pathway which is activated by TNF and IL-1, two cytokines which can inhibit the growth of some mammary tumor lines. This pathway uses a subfamily of the ERKs, the stress-activated protein kinases (SAPKs). SAPKs are the dominant kinases responsible for activation of the transactivation function of the transcription factor c-Jun. As such, the SAPKs relay TNF signals to the cell nucleus, thereby regulating gene expression in response to TNF. During the past year, we have identified SAPK/ERK kinase-1 as a kinase which activates the SAPKs in situ, and MAPK/ERK kinase-kinase (MEKK1) as an in situ SAPK/ERK kinase (SEK1) activator. insofar as SEK is a novel member of the MEK family and MEKK 1 is a homolog of yeast STE11, the SAPK pathway is homologous to yeast MEKK-MEK-ERK signaling modules. These modules are themselves regulated by members of the STE20 family of protien kinases. Consistent with this, we observe that the SAPK pathway can be activated by the mammalian STE20 homolog, GC kinase. GC kinase is also activated in situ by TNF. Thus, TNF activates a pathway consisting of GC kinase-?-MEKK-SEK1-SAPK-c-Jun. We propose that this pathway may mediate inhibition of mammary cell growth in response to TNF.

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FOREWORD

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INTRODUCTION

We have elucidated a second mammalian signaling pathway which responds primarily to stress and the inflammatory/pro-apoptotic cytokines TNF-α and IL-1. This finding has implications for the understanding of the inflammatory response and controlled cell death. This pathway, uses a member of the extracellular signal-regulated protein kinase family, the p54 heat-activated protein kinases (HAPKs, now renamed SAPKs for stress-activated protein kinases) as a multifunctional effector which, among other things, phosphorylates and activates transcription factors. Specifically, our past findings indicate that the SAPKs are the dominant c-Jun kinases, as well as ATF-2 kinases, activated by TNF-α. Thus, one purpose of the SAPKs is the relaying of inflammatory/stress/apoptotic signals to the nucleus. As such, the SAPKs have an important role in the regulation of gene expression in response to cellular/physiologic stress and apoptotic agonists. The implication of TNF-α as a SAPK agonist indicates that this important breast cell growth inhibitor likely uses the SAPKs to influence gene expression in breast cells.

The SAPKs are part of a multi-tiered protein kinase cascade which is strikingly similar to signaling modules in yeast. In these pathways, members of the *STE20* family of protein kinases phosphorylate and activate a cassette of signaling kinases consisting of a MEK-kinase (MEKK) which activates a MAPK/ERK kinase (MEK) which, in turn, activates an ERK. ERKs serve to distribute the signal to the downstream components brought to bear in response to the particular extracellular stimulus (1). Consistent with the homology between yeast and mammalian signaling mechanisms, we have now identified components in the SAPK pathway which lie upstream of the SAPKs and identified them as members of the MEK, MEKK and *STE20* kinase families.

As outlined in the original proposal, it is the aim of this project to characterize the regulation, and cellular role of the SAPKs. These studies have been be divided into four major areas.

1) *What are the cellular agonists which activate the SAPKs?*

2) *What are the immediate upstream activators of the SAPKs?* A primary goal of this project will be the identification of protein kinase cascades capable of activating the SAPKs *in situ.*

3) *Do the SAPKs activate MAPKAP-kinase-2?.* The true *in vivo* substrates of the SAPKs, aside from c-Jun, are not known. These studies will identify protein substrates of the SAPKs whose physiologic function is changed by SAPK phosphorylation.

4) *Do the SAPKs have a role in mammary tissue proliferation?*

BODY: 8/94-8/85

During the past year, the investigations in this laboratory have focussed primarily on the elucidation of the protein kinase components upstream of the SAPKs and the downstream targets of the SAPKs. The following conclusions were drawn using the techniques described:

1) *MAPKAP kinase-2 (MAPKAP-K2) is not a SAPK substrate.* Using purified proteins (MAPKAP-K2 was kindly provided by Prof. Philip Cohen, University of Dundee) we have demonstrated that SAPK cannot phosphorylate and activate MAPKAP-K2. Fig. 1 illustrates these results.

Effect of HepG2 cell SAPKs on MAPKAP kinase-2

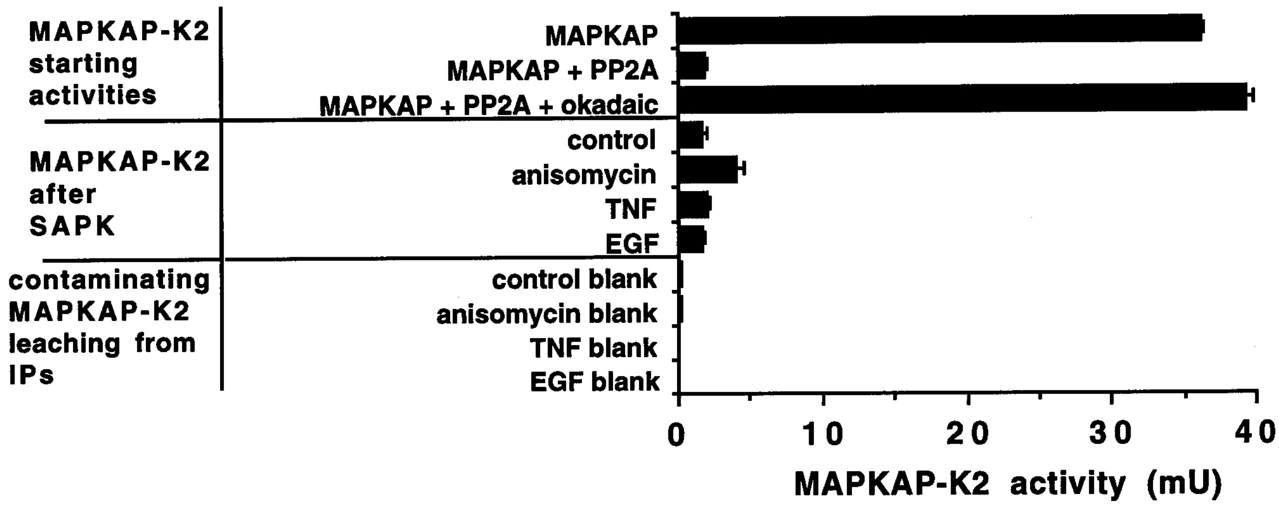

Fig. 1. SAPK does not reactivate phosphatase-2A-inactivated MAPKAP-K2. SAPK was immunoprecipitated from HepG2 cells treated with the lignads indicated and incubated with purified MAPKAP-K2 which had been inactivated with phosphatase-2A as indicated. After centrifugation to remove the SAPK beads, MAPKAP-K2 was assayed as described.

2) *Identification of SAPK/ERK Kinase-1 (SEK1) as an Upstream Activator of the SAPKs.* Using recombinant SAPK as a substrate, the PI-s laboratory has identified a novel member of the mitogen-activated protein kinase/extracellular signal regulated kinase-kinase (MEK) family, SEK1, as an upstream activator of the SAPKs. SEK1 was cloned in the laboratory of Dr. Leonard Zon, with whom we collaborated in these studies. SEK1 is completely specific for the SAPKs, being unable to activate the MAPKs *in situ* or *in vitro*. SEK1 activity is activated preferentially by the same stress stimuli which activate the SAPKs *in situ*. These results further illustrate the segregation between the mitogen-activated Ras/MAPK pathway and the SAPK stress-regulated pathway (2,3); and support the contention that mammals, like yeast possess multiple, homologous signaling pathways which respond to distinct types of extracellular stimuli. Fig. 2 illustrates activation of the SAPKs *in situ* by purified SEK1 (1-3).

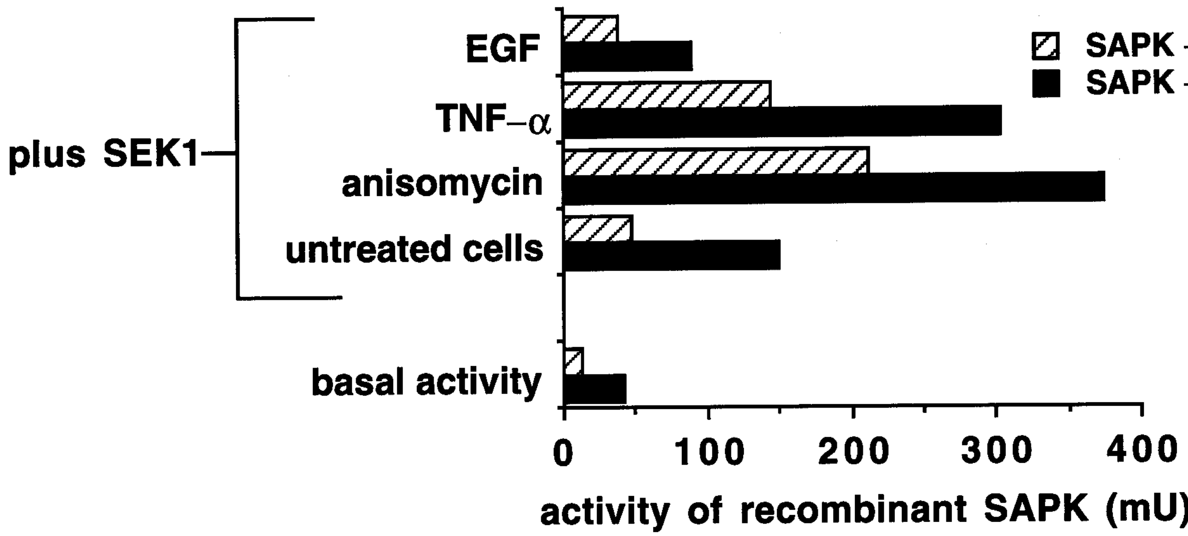

Fig. 1. Activation of SAPKs by SEK1. SEK1 was purified from transfected cells and assayed for activation of purified recombinant SAPKs p54αI and p54βI. Assays were performed as in Sánchez, *et al.* (1994).

These studies were published in: Sánchez, I *et al.* (1994) *Nature.* **372**, 794-798.

3) *Identification of MEK-kinase-1 (MEKK1) as an Upstream Activator of SEK1 and the SAPK Pathway.* MEKK1 is a mammalian homolog of the yeast MEK activators Ste11 and Byr2 (4). Originally, MEKK1 was thought to act as a mitogen-activated, Raf-1-independent mechanism of MEK activation (4). Using an inducible MEKK1 construct, in collaboration with Dr. Dennis Templeton, we have demonstrated that MEKK1 preferentially activates the SAPK pathway, via activation of SEK1 (5). These studies, further delineate the segregation of the SAPK and MAPK pathways and strikingly illustrate the homology between yeast and mammalian signaling mechanisms. Fig. 3 illustrates *in situ* activation of SAPK upon coexpression with MEKK1.

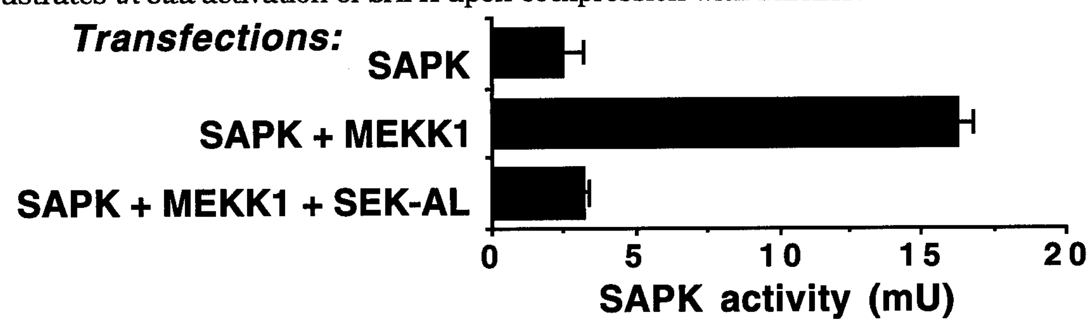

Fig. 3. Coexpression with MEKK1 activates SAPK. This activation is blocked by triple transfection with a nonactivatable SEK1 mutant.

Fig. 4 illustrates the ability of purified MEKK1, *in vitro* to activate the SAPK activating activity of SEK1.

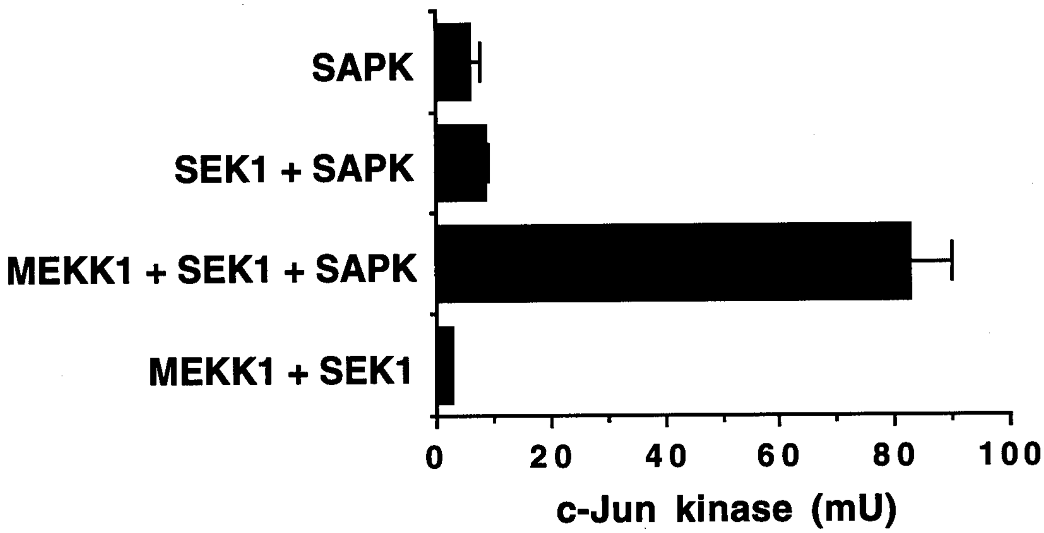

Fig. 4. MEKK1, SEK1 and SAPK-p54αI were expressed as GST fusion proteins in *E. coli* and purified by glutathione affinity chromatography. Purified MEKK1 was then tested for its ability to activate SEK1 SAPK activating activity in this coupled assay.

These studies were published in: Yan, M. *et al.* (1994) *Nature.* **372**, 798-800.

4) *Identification of GC Kinase, a Mammalian* STE20 *Homolog, as an Activator of the SAPK Pathway.* In yeast, the MEKK → MEK → ERK core kinase modules are thought themselves regulated by members of the *STE20* family of protein kinases (1). GC kinase is a ubiquitously expressed mammalian *STE20* homolog which, in lymphoid follicles is thought to participate in B cell maturation (6). GC kinase was cloned in Dr. John Kehrl's laboratory and he has generously provided us with the clone. We have now shown that GC kinase is a potent, specific *in situ* activator of the SAPK pathway, and overexpression of the GC kinase activates both SEK1 and the SAPKs. Figs. 5 and 6 illustrate activation of SAPK and SEK1, respectively upon coexpression with GC kinase. In Fig. 7, it is clear that GC kinase does not activate two other ERK pathways, the p42/p44 MAPK or p38/mpk2 pathway (7).

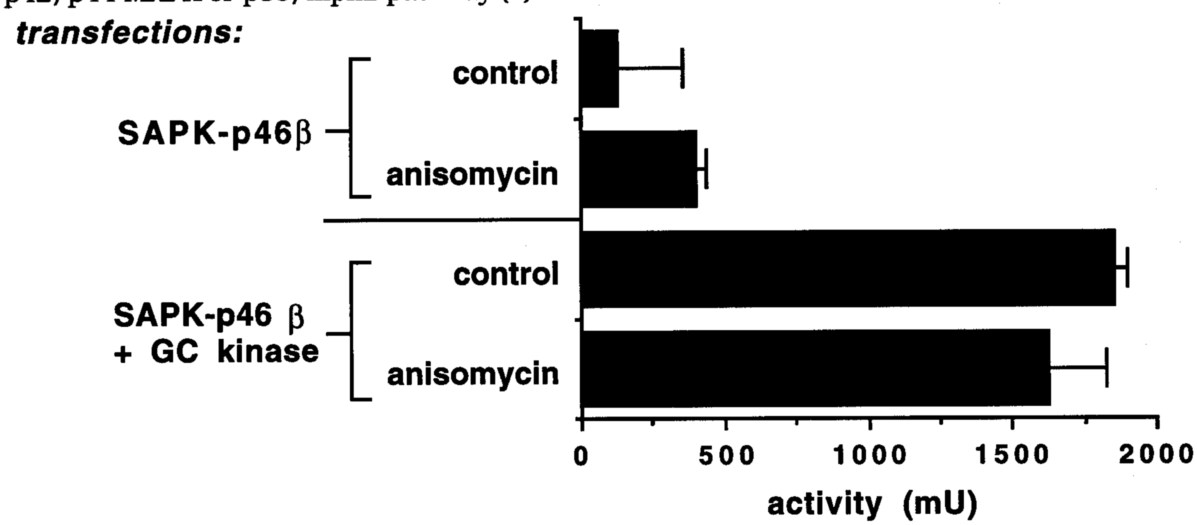

Fig. 5. Activation *in situ* of SAPK by GC kinase. 293 cells were transfected with HA-tagged SAPK and GC kinase or the cognate empty plasmid. SAPK was immunoprecipitated and assayed.

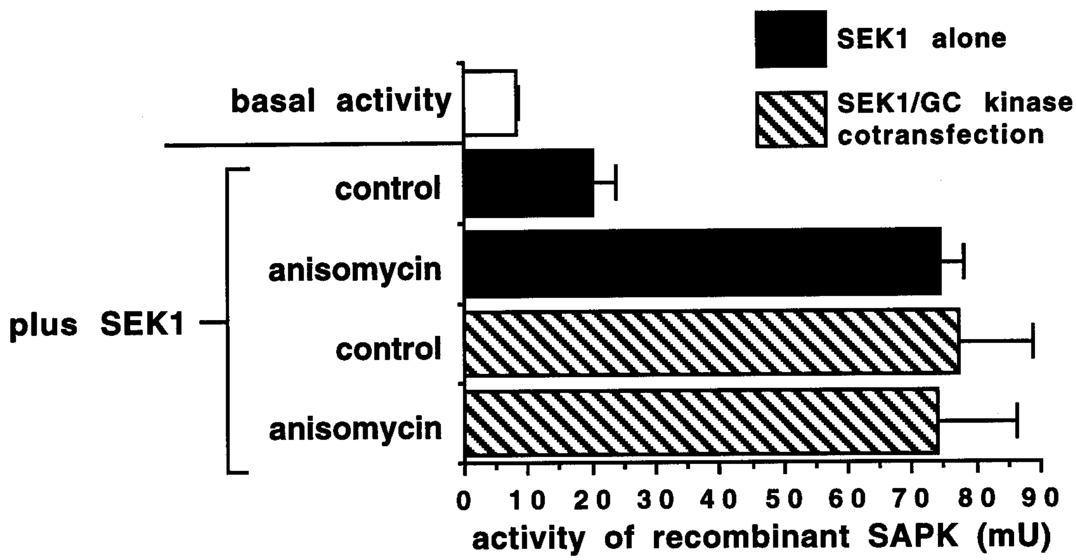

Fig. 6. Activation *in situ* of SEK1 upon coexpression with GC kinase. 293 cells were transfected with GST-tagged SEK1 and either GC kinase or cognate empty plasmid. SEK1 was purified by glutathione agarose affinity chromatography and assayed for activation of SAPK.

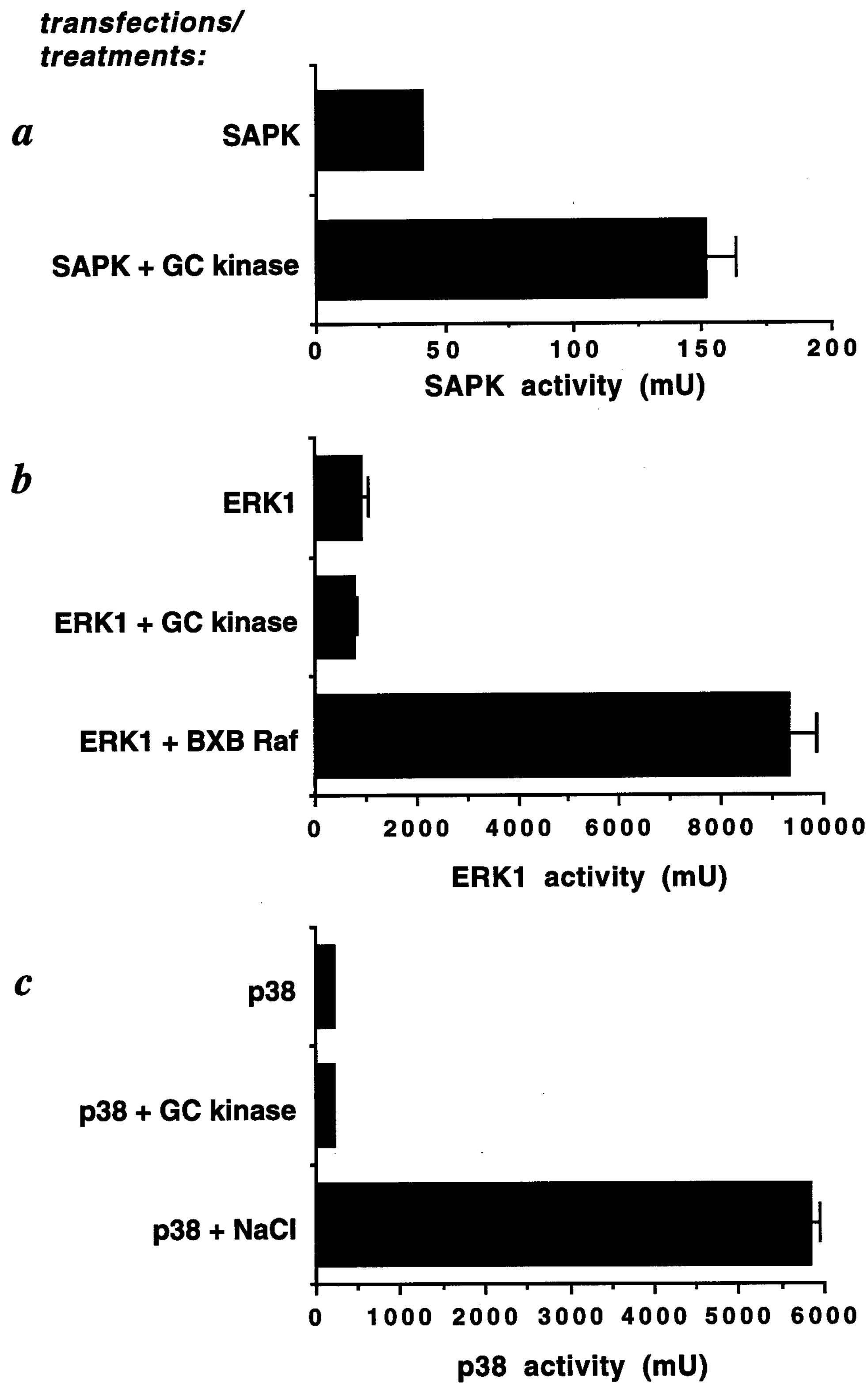

Fig. 7. Coexpression with GC kinase in COS cells activates the SAPK pathway (part *a*) but does not activate the p42/p44 MAPK pathway (part *b*, ERK1) or the p38 pathway (part *c*) in spite of the fact that these two pathways are intact and can be activated, respectively by oncogenic Raf-1 (BXB-Raf) or by hyperosmotic shock (NaCl).

We have also obtained preliminary data indicating that GC kinase is in part regulated by an inhibitor which is present in limiting amounts in the cell and which interacts with the C-terminal noncatalytic domain of GC kinase. In support of this we observe that overexpression of the GC kinase C-terminus activates the SAPK pathway likely by competing away this inhibitor.

These studies have been submitted for publication as: Pombo, C.M., *et al. Nature.* submitted.

The work outlined above, as well as related projects in other areas have resulted in the following publications during the period 8/19/94-8/28/95. This level of productivity would not have been possible without the Army funding. No meeting abstracts were published or submitted.

Kyriakis, J.M. and Avruch, J. (1995) S6 kinases and MAP kinases: Sequential intermediates in insulin/mitogen-activated protein kinase cascades. In: *Protein Kinases: Frontiers in Molecular Biology*, J.R. Woodgett, ed., Oxford University Press: Oxford.

Avruch, J., Zhang, X.-f. and Kyriakis, J.M. (1994) Raf meets Ras: closing a frontier in signal transduction. Trends Biochem. Sci. 19: 279-283.

Pombo, C.M., Bonventre, J.V., Avruch, J., Woodgett, J.R., Kyriakis, J.M. and Force, T. (1994) The stress-activated protein kinases are major c-Jun amino-terminal kinases activated by ischemia and reperfusion. J. Biol. Chem. 269: 26546-26551.

Bird, T.A., Kyriakis, J.M., Tyshler, L., Gayle, M., Milne, A. and Virca, G.D. (1994) Interleukin-1 activates p54 mitogen-activated protein (MAP) kinase-stress-activated protein kinase by a pathway that is independent of p21ras, Raf-1 and MAP kinase kinase. J. Biol. Chem. 269: 31836-31844.

Sánchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. and Zon, L.I. (1994). Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature. 372: 794-798.

Yan, M., Dai, T., Deak, J., Kyriakis, J.M., Zon, L.I., Woodgett, J.R., and Templeton, D.J. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature. 372: 798-800.

Pombo, C.M., Kehrl, J.H., Sánchez, I., Katz, P., Avruch, J., Zon, L.I., Woodgett, J.R. Force, T., and Kyriakis, J.M. (1995) Activation of the SAPK pathway by GC kinase, a human *STE20* homolog. Nature, Submitted.

Personnel:

The following personnel were paid from this grant during the period 8/19/94-8/28/95:

John M. Kyriakis, Ph.D. Principal Investigator
Irma Sánchez, Ph.D. Post Doctoral Research Fellow (from 7/1/95)
Árpád Molnár, M.D. Post Doctoral Research Fellow (from 4/1/95)
Anna Maria Forte, M.S. Research Technician

No graduate degrees were received during the period 8/19/94-8/28/95.

CONCLUSIONS: 8/94-8/95

With the conclusion that the SAPKs are part of a core signaling module which includes MEKK1 and SEK1, and that this module may be regulated *in situ* by GC kinase and other *STE20* homologues, it appears that the SAPK signaling pathway is strikingly homologous to yeast signaling mechanisms (reviewed in 1). In yeast, multiple signal transduction mechanisms are regulated by modules consisting of members of the MEKK, MEK and ERK families of protein kinases arranged MEKK → MEK → ERK. These modules are, in turn regulated by an as yet uncharacterized mechanism, by members of the *STE20* family of protein kinases (1). Accordingly, we plan to pursue the following studies for the forthcoming year:

1) *Continuation of the Yeast Two Hybrid Screen for SAPK Substrates.* Inasmuch as MAPKAP-K2 is not a SAPK substrate, we do not plan to pursue further MAPKAP-K2 experiments. Instead, we are continuing to characterize 103 clones isolated in a yeast two hybrid screen for SAPK-interacting polypeptides. We anticipate that many of these will be additional SAPK substrates.

2) *Identification of Additional MEKK Family Members which can Activate the SAPKs.* SAPKs may be part of multiple MEKK MEK ERK signaling modules. The mammalian MEKKs are a large family of protein kinases, of which at least six members exist. We are currently cloning additional MEKKs for use in expression studies to determine if these activate the SAPKs.

3) *Characterization of the Regulation and Substrates of GC Kinase.* We do not know the mechanism by which GC kinase activates the SAPK pathway. Accordingly, we are setting up a yeast two hybrid screen in order to identify polypeptides which interact with GC kinase. We anticipate that some of these will be GC kinase substrates and others will be species involved in GC kinase regulation.

4) *Identification of Additional* STE20 *Homologs which Can Activate the SAPK Pathway.* We are using a PCR-based approach to clone additional members of the mammalian Ste20 family which can activate the SAPK pathway. We have already isolated one such kinase PK-1, which is currently being tested as an activator of the SAPK pathway.

We are confident that these studies will be significant to the pathology of breast cancer. Specifically, the SAPKs are potently activated by TNF and IL-1. Inasmuch as c-Jun is a principal target of TNF action and is a SAPK substrate, the SAPKs play an important role in TNF and IL-1 action. Moreover, these cytokines have been shown to be potent inhibitors of breast cell growth. By understanding the SAPK pathway, and identifying all of the components in the SAPK signaling network, it will be possible to design more specific and effective inhibitors of breast cell neoplasias based on the SAPK pathway.

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3) Sánchez, I, Hughes, R.T., Mayer, B., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M. & Zon, L.I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature. 372: 794-798.

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