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

Understanding the biochemical mechanisms of apoptosis is important for revealing cancer biology and for improving cancer therapies. The c-Jun N-terminal kinase (JNK) pathway participates in cellular responses to both environmental stresses and mitogenic signals. The involvement of JNK in both mitogenic and apoptotic signaling implies that a subtle regulatory mechanism must exist for the signaling decision. Our studies reveal that the duration of JNK induction may determine cell fate. JNK is involved in cellular signaling during apoptosis induced by various agents including γ-radiation, UV-radiation, anti-carcinogenic isothiocyanates, and retinoid analog N-(4-hydroxyphenyl) retinamide (4-HPR) in various cancer cells. Interfering with the JNK pathway suppressed the induction of apoptosis. JNK activation by apoptotic stimuli can be caspase-dependent or independent. Oxidative stress induces JNK activation through the down-regulation of JNK-inactivating phosphatases. In conclusion, our study reveals the importance of the JNK pathway in apoptotic signaling. The results of this study provide important information for the studies of oncogenesis and may facilitate the development of strategies for the prevention and treatment of prostate and other cancers.

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INTRODUCTION

Disorders in apoptosis occur in tumorigenesis and play an important role in developing resistance to radiation and chemotherapy in tumor cells. The c-Jun N-terminal kinase (JNK) pathway is involved in and required for apoptotic signaling induced by various stimuli, including anticancer therapeutic agents. JNK family members belong to the mitogen-activated protein kinase (MAPK) superfamily (1,2). The JNK pathway responds to diverse stimuli such as mitogens, fluid shearing, pro-inflammatory cytokines, and environmental stresses including various apoptotic stimuli (2). Like the MAPK pathway in yeast, the mammalian JNK pathway consists of a four-kinase module — MAP4K, MAP3K, MAP2K, and JNK (MAPK). Currently, two MAP2Ks, MKK4/SEK1 and MKK7, and multiple MAP3Ks and MAP4Ks are known to activate JNK (2). Activated JNK can be down-regulated by JNK-specific phosphatases. JNK activation is observed in apoptosis induced by a variety of stimuli in a variety of cell types. Interference with the JNK pathway suppresses apoptosis (1,2). Murine embryonic fibroblasts from *jnk1/jnk2* deficient mice are resistant to apoptosis induced by UV-C, ansiomycin, and mitomycin C (3). These results indicate the importance of the JNK pathway in apoptotic signaling. It has been shown that JNK may induce apoptosis by enhancing the expression of Fas ligand. Cytochrome c release from mitochondria is absent in UV-treated *jnk1/jnk2* deficient cells, suggesting that JNK is required for cytochrome c release (3). Currently, the mechanism by which JNK is activated by apoptotic stimuli is unclear. This study is to reveal the molecular mechanism of JNK-mediated apoptotic signaling. Results derived from this study may shed light on the underlying mechanisms of resistance to cancer therapeutic drug and radiation treatment.

BODY

The first two sections are the summaries of the studies that were initiated before July 1, 1999, and were completed during the funding period. Although these studies were not described in the proposal, both studies are related to the proposed research and the detail and related publications were included in the 2000 Annual report.

1. JNK mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide (4-HPR) in prostate cancer cells *(These results were published in Ref. (4))*

N-(4-hydroxyphenyl) retinamide (4-HPR), a retinoic acid analog, induces apoptosis in several cell types. The mechanism by which 4-HPR initiates apoptosis remains poorly understood. We examined the effects of 4-HPR on two prostate carcinoma cell lines, LNCaP (an androgen-sensitive, $p53^{+/+}$ cell line) and PC-3 (an androgen-insensitive, $p53^{-/-}$ cell line). 4-HPR caused sustained c-Jun N-terminal kinase (JNK) activation and apoptosis in LNCaP cells but not in PC-3 cells at the dosages tested. Activation of JNK by 4-HPR was independent of caspases, since a pan-caspase inhibitor failed to suppress JNK activation. Ultraviolet C and γ radiation induced JNK activation in both LNCaP and PC-3 cells, suggesting that the failure of PC-3 cells to respond to 4-HPR was due to defects upstream of the JNK pathway. Furthermore, γ radiation-induced JNK activation was suppressed by an antioxidant, but 4-HPR-induced JNK activation was not, indicating that these two stimuli induced JNK activation through different mechanisms. Forced expression of JNK1, but not a JNK1 mutant, caused apoptosis in both LNCaP and PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. 4-HPR-induced apoptosis in LNCaP cells was suppressed by curcumin, which inhibits JNK activation. Expression of dominant-negative mutants in the JNK pathway also inhibited 4-HPR-induced apoptosis in HEK293 cells. Taken together, these results suggest that the JNK pathway mediates 4-HPR-induced apoptotic signaling.

2. **Caspase-mediated cleavage of JNK-activating kinase HPK1** *(these results were published in Ref. (5))*

Activation of c-Jun N-terminal kinase (JNK) by Fas ligation is caspase-dependent, suggesting that caspases may regulate activators of the JNK pathway. We found that an upstream activator of JNK, hematopoietic progenitor kinase 1 (HPK1), was cleaved during apoptosis. Cleavage of HPK1 was blocked by peptide inhibitors for caspases. HPK1 was efficiently processed by recombinant caspase 3 *in vitro*. A conserved caspase recognition site, DDVD (amino acid 382-385), was found in the HPK1 protein sequence. By testing HPK1 proteins with *in vivo* and *in vitro* cleavage assays, we showed that aspartic acid residue 385 is the target for caspases. HPK1 cleavage separated the amino (N)-terminal kinase domain from the carboxyl (C)-terminal regulatory domain, and enhanced HPK1 kinase activity. Unlike the full-length HPK1, the N-terminal cleaved product failed to bind adaptor molecules Grb2 (growth factor receptor-bound protein 2) and Crk (CT10 regulator of kinase). The C-terminal fragment, although having three proline-rich domains, bound to Grb2 and Crk less efficiently than the full-length HPK1 protein. Taken together, the cleavage of HPK1 by caspase profoundly changed its biochemical properties.

3. Induction of JNK by Mitogenic and Apoptotic Stimuli in Prostate Cancer Cells *(these experiments are related to specific aim #1 in the Statement of Work)*

We found that the induction of JNK in response to mitogenic and apoptotic signals in prostate cancer LNCaP cells have different activation patterns, transient versus persistent, respectively. We treated LNCAP cells with either PMA or epidermal growth factor (EGF). Both PMA and EGF induce an immediate and transient JNK activation in LNCaP cells. The kinase activity increased in 15 min and decreased to basal levels in 90 min. Androgen treatment did not induce significant JNK activation in LNCaP cells. LNCAP were also treated with various apoptotic agents, and examined for endogenous JNK activation. Among the agents tested, γ radiation, UV-C, 4-HPR, and 5-FU showed strong JNK inducing ability. All of the apoptotic agents induced persistent JNK activation, which followed by apoptotic cell death as determined by morphological change and nuclear staining of the cells. These data reveal that mitogenic agents induced transient JNK activation in LNCaP cells, while apoptotic agents induced persistent JNK activation. These results are consistent with our previous observation in T cells, and further support our hypothesis that duration of JNK activation may determine cell fate.

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa*, gives the golden-yellow color and unique flavor to curry. Previously, we found that curcumin may affect the JNK pathway by interfering with the signaling molecule(s) at the same level or proximally upstream of the MAPKKK level (6). Curcumin treatment suppresses the proliferation of LNCaP cells in response to serum. Curcumin also suppress apoptosis induced by 4-HPR in LNCaP cells (4). These data suggest that the JNK pathway is required for both proliferation and apoptosis in prostate cancer cells.

4. Post-radiation treatment of curcumin, an inhibitor for JNK activation, fails to terminate JNK activation *(this work is related to specific aim #1 in the Statement of Work)*

Since curcumin is an effective inhibitor for JNK activation by suppressing upstream kinases (6), we tested whether curcumin can be used to terminate JNK activation induced by apoptotic signals. If so, the association of duration of JNK induction and apoptosis can be determined. LNCAP cells were treated with UV-C. Twenty min post-irradiation, cells were incubated with or without curcumin. Although pre-incubation of curcumin inhibited JNK activation by all of the agonists tested (6). **Post-radiation treatment of curcumin failed to suppress the intensity or the duration of JNK activation.** This result suggests that once JNK has

been activated, the activities of upstream kinase were not required for the sustained JNK activation, because of suppressing the upstream kinase by curcumin is not sufficient to turn off the JNK activation.

From this study, we conclude that curcumin cannot be used to modulate the duration of JNK activation as proposed in the research proposal (Aim 1). However, a JNK specific inhibitor was recently reported. This inhibitor may be used to control the duration of JNK activation.

5. The role of Fas-Fas ligand in JNK-mediated apoptosis in prostate cancer cells *(this work is related to specific aim #2 in the Statement of Work)*

Previously, we have shown Fas expression induced by apoptotic stimuli is a p53-dependent phenomenon, but can be dissociated from JNK activation (7). Other investigators have reported that JNK activation leads to the expression of Fas ligand. Using various methods, we did not detect expression of Fas ligand in LNCaP prostate cancer, even in the presence of strong JNK activation. To examine the relationship between Fas/Fas ligand and JNK-mediated apoptosis, we established radiation-resistant LNCaP cells by exposing parental LNCaP cells to lethal doses of γ radiation (also see Section 7). These radiation-resistant LNCaP cells did not respond to γ radiation with JNK activation and apoptotic cell death, which is consistent with the finding that JNK is required for γ radiation-induced apoptosis. However, these radiation-resistant cells were still sensitive to apoptosis induced by Fas ligation. Currently, the collective data indicate that the Fas-Fas ligand pathway is not required for radiation-induced and/or JNK-mediated apoptosis (7). We are establishing LNCaP cells that are resistant to Fas-mediated apoptosis. We will test whether those cells are sensitive to JNK-mediated apoptosis. The radiation-resistant LNCaP cells will be used to identify the pathway(s) that are involved in JNK activation by γ radiation.

Changes of Experimental Approaches: *The Aim 3 (Tasks 3a –3d) of the granted proposal was to identify the upstream kinases that mediate JNK activation by apoptotic stimuli. The proposed candidate was apoptosis signal-regulating kinase 1 (ASK1). Our preliminary studies and recent reports by others revealed that ASK1 might not be the major activator of JNK-mediated apoptotic signaling; hence the experimental approaches have been modified. The new lines of research have lead to significant results, which were described in Sections 6 and 7.*

6. Oxidative stress induces JNK activation via down-regulation of JNK-inactivating phosphatases *(this work was published in Ref. (8))*

Apoptotic stimuli (e.g., γ radiation and chemotherapeutic agents) often induce the production of reactive oxygen species in cells (9-12). Oxidative stress activates the c-Jun N-terminal kinase (JNK) pathway (reviewed in 2). However, the exact mechanisms by which ROS activate JNK are unclear. We found that the ability of hydrogen peroxide (H_2O_2) to induce JNK activation varied in different cell types. Pyrrolidine dithiocarbamate (PDTC), a presumed antioxidant (13,14), induced JNK activation on its own and enhanced JNK activation by H_2O_2 in many cell types, including Jurkat, HEK293, and LNCaP and Tsu-Pr1 prostate cancer cells. The activation of JNK by PDTC, in the presence or absence of exogenous H_2O_2, was dependent on its chelating ability to metal ions, most likely copper ions. These data suggest that copper ions imported by PDTC decompose H_2O_2 to form hydroxyl radicals and cause cellular damages and JNK activation. Our data also reveal a necessity to carefully evaluate the pharmacological and biochemical properties of PDTC (8).

Despite the strong JNK-activating ability, H_2O_2 plus PDTC did not induce significant activation of the upstream kinases, SEK1/MKK4 and MKK7. However, the JNK inactivation rate was slower in cells treated with H_2O_2 plus PDTC compared with the rate in cells treated with ultraviolet C (UV-C). Treatment of H_2O_2

plus PDTC significantly decreased the expression levels of a JNK phosphatase, M3/6 (also named hVH-5), but not the levels of other phosphatases (PP2A and PP4). In contrast, UV-C irradiation did not cause the down-regulation of M3/6. In addition, H_2O_2 also down-regulated the expression of another JNK phosphatase MKP-1. These results suggest that JNK activation by H_2O_2 plus PDTC resulted from the down-regulation of JNK phosphatases. This study reveals a novel mechanism of JNK activation by oxidative stress, which may play an important role in apoptotic signaling induced by many anticancer agents.

7. Search the critical factor(s) for JNK activation by studying defects in JNK activation in genotoxic-resistant LNCaP clones *(this work has not been published)*

ATM has been shown to be required for both cell cycle arrest and JNK activation induced by genotoxic agents (reviewed in 2). However, we found that JNK can be activated only in cells exposed to lethal doses of γ radiation, but ATM activation and cell cycle arrest can be observed in cells exposed to sublethal or lethal doses of γ radiation. This result suggests that ATM activation is not sufficient for JNK activation, and that an additional factor (or factors) is required for JNK activation. If additional factors are involved in determining whether JNK-mediated apoptotic signaling can be activated in cells exposed to genotoxicity, certain genetic changes should be able to block JNK activation without affecting the events of cell cycle arrest. Mutant cells that are defective in JNK activation and apoptosis, but retain normal cell cycle arrest in response to genotoxicity, will provide useful information about JNK-mediated apoptotic signaling. We have established a mixed population of LNCaP cells (LNCaP-IR10) that were selected by resistance to γ radiation-induced apoptosis (Fig. 1). We have isolated several individual clones from this mixed population and are examining their responses to various genotoxic stimuli (γ radiation, 5-FU, and cisplatin). We expect that these resistant clones will have distinct properties in response to genotoxic agents. Clones that display cross-resistance to multiple genotoxic agents will be given higher priority for detailed study.

The isolated clones may be defective in both cell cycle arrest and apoptosis induction (Type I), or be defective in only apoptosis but not in cell cycle arrest (Type II and Type III; Fig. 1). We will focus our study on the second group of cells. In this group of cloned cells (only defective in the apoptotic response), we predict that some (Type II) will have JNK activation in response to γ radiation and some (Type III) will not. Those cells that are defective in JNK activation (Type II) will be examined for potential defects in JNK upstream signaling in this sub-aim.

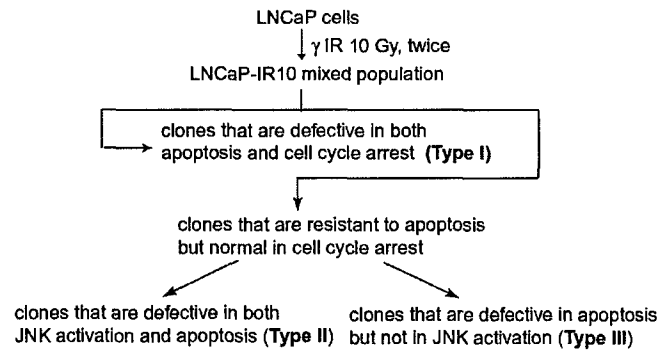

Fig. 1. Selection of LNCaP cells that are resistant to genotoxic stimuli.

We have characterized one LNCaP cell clone (named LNCaP-IR10.1) that is defective in JNK activation and resistant to apoptosis induced by γ radiation and 5-FU treatment, but is functional in the induction of p21 expression. These data suggest that cells can lose the ability to undergo apoptosis, but still retain other DNA damage responses (cell cycle arrest and/or DNA repair). Because JNK can be activated by non-genotoxic stimuli in LNCaP-IR10.1 cells, it indicates that the JNK-signaling module is still responsive, and that the defects are more likely to be in the upstream regulators that mediate JNK activation by genotoxicity. We are in the process of establishing more Type II γ radiation-resistant clones with the same or similar properties. These γ radiation-resistant clones will be used to study their defects in cellular signaling from DNA damage to the JNK pathway. We anticipate various genetic defects may lead to the unresponsiveness of JNK to γ radiation in

different cloned cells. These γ radiation-resistant cells will be used to examine the stress signaling that is critical for JNK-mediated apoptosis but not for cell cycle arrest and other genotoxic responses.

In addition to the experiments described, the established LNCaP resistant clones will be very useful in identification of downstream events that mediate γ radiation- and JNK-induced apoptosis. They can also be used to screen for therapeutic agents that are effective on genotoxic-resistant cancers. These cell clones will also be useful for the scientific community for additional applications. For example, we are currently collaborating with researchers at Baylor College of Medicine to test viral-based and chemical therapeutic agents for cancer using the established genotoxic-resistant clones as experimental models.

KEY RESEARCH ACCOMPLISHMENTS

1. Showing that JNK is involved in and required for N-(4-hydroxyphenyl)retinamide-induced apoptosis in LNCaP cells (Ref. 4)
2. Showing that p53 tumor suppressor gene is not required for JNK-mediated apoptosis (Ref. 4)
3. Identification of hematopoietic progenitor kinase 1 (HPK1), a JNK-activating kinase, as a substrate for caspase 3 (Ref. 5)
4. Showing that down-regulation of JNK-inactivating phosphatases is a mechanism of JNK activation by oxidative stress (Ref. 8)
5. Establishment of γ radiation-resistant LNCaP cells.

REPORTABLE OUTCOMES

Meeting Abstracts *(attached in the 2000 Annual Report)*

1. Spore in Prostate Meeting, Mar. 23, 2000, Baylor College of Medicine, Houston; presentation entitled "The c-Jun N-terminal kinase pathway and apoptotic signaling in prostate cancer"
2. Keystone Symposium entitled "Advances in Human Breast and Prostate Cancer" mar. 19-24, 2000, Lake Tahoe, Nevada; poster abstract (#203) entitled " c-Jun N-terminal kinase-mediated apoptotic signaling in prostate cancer"

Publications *(#1,2,4 were attached in the 2000 Annual Report; #3 and 5 were attached in this report)*

1. Y.-R. Chen, C. F. Meyer, B. Ahmed, Z. Yao, and T.-H. Tan (1999) Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). *Oncogene* **18**, 7370-7377
2. Y.-R. Chen, G. Zhou, and T.-H. Tan (1999) c-Jun N-terminal kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide (4-HPR). *Mol. Pharmacol.*, **56**, 1271-1279
3. Y.-R. Chen and T.-H. Tan (1999) Mammalian c-Jun N-terminal kinase pathway and STE20-related kinases (Review). *Gene Therapy and Molecular Biology* 4, 83-98
4. Y.-R. Chen and T.-H. Tan (2000) The c- Jun N-terminal kinase pathway and apoptotic signaling (Review). *Int. J. Oncol*, **16**, 651-662
5. Y.-R. Chen, A. Shrivastava, and T.-H. Tan (2001) Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. *Oncogene*, 20, 367-374

Awards

Travel award (Advance in human breast and prostate cancer, Keystone symposium 2000; provided by NIH Grant#1 R13 CA85280-01)

CONCLUSION

The c-Jun N-terminal kinase (JNK) pathway participates in cellular responses to both environmental stresses and mitogenic signals. The involvement of JNK in both mitogenic and apoptotic signaling implies that a subtle regulatory mechanism must exist for the signaling decision. Our studies reveal that the duration of JNK induction may determine cell fate. The mechanisms that regulate the duration of JNK activation are currently studied. We found that JNK is involved in cellular signaling during apoptosis induced by various agents including γ-radiation, UV-radiation, anti-carcinogenic isothiocyanates, and retinoid analog N-(4-hydroxyphenyl) retinamide (4-HPR) in various cancer cells (4,15,16). Interfering with the JNK pathway suppressed the induction of apoptosis. JNK activation by apoptotic stimuli can be caspase-dependent or independent. Different apoptotic stimuli induce JNK activation through distinct mechanisms ((5); and reviewed in 2). We found that oxidative stress is a major cause of JNK activation in apoptosis induced by several agents (8,16). Oxidative stress induces JNK activation through the down-regulation of JNK-inactivating phosphatases (8). Radiation-resistant prostate cancer cells have been established for identification of critical factors that are required for JNK activation in response to genotoxicity. These cells will also be used to identify potential therapeutic agents for genotoxic-resistant prostate cancer.

In conclusion, our study reveals the importance of the JNK pathway in apoptotic signaling (reviewed in 2). Results derived from this study may shed light on the underlying mechanisms of resistance to cancer therapeutic drug and radiation treatment. New chemopreventive or therpeutic agents can also be developed based on the revealed molecular mechanisms of cellular signaling. Therefore, this research may have an important impact on the development of strategies and therapeutic agents for the prevention and treatment of prostate and other cancers.

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APPENDICES *(#1, pages 11-26; #2, pages 27-34)*

1. Y.-R. Chen and T.-H. Tan (1999) Mammalian c-Jun N-terminal kinase pathway and STE20-related kinases (Review). *Gene Therapy and Molecular Biology* 4, 83-98
2. Y.-R. Chen, A. Shrivastava, and T.-H. Tan (2001) Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. *Oncogene*, 20, 367-374

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Mammalian c-Jun N-terminal kinase pathway and STE20-related kinases

Review Article

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Summary

The c-Jun N-terminal kinases (JNKs) belong to a subgroup of mitogen-activated protein kinases (MAPKs) that are activated by environmental stress, proinflammatory cytokines, and mitogenic stimuli in mammalian cells. Studies on the JNK pathway in mammalian cells demonstrate that JNK regulates the transcriptional activities of many transcription factors, and that JNK is required for the regulation of cell proliferation and apoptosis. Studies on *jnk*-deficient mice reveal that JNK is involved in the response to immunological stimuli and in embryonic morphogenesis. JNK, as other MAPKs, is regulated by a kinase cascade. JNK activation is mediated by dual phosphorylation on the motif, Thr-Pro-Tyr. To date, this phosphorylation is known to be mediated by the MAPK kinases (MAP2Ks), MKK4 and MKK7. MKK4 and MKK7 are activated by MEKK1 and other MAPK kinase kinases (MAP3Ks). The MAPK kinase kinase kinases (MAP4Ks) including HPK1, GCK, and homologous kinases, which have a kinase domain related to yeast STE20, can activate the JNK signaling cascade. These mammalian STE20-related MAP4Ks may be involved in integrating various stimuli to the JNK cascade. The signaling specificity of mammalian JNK pathway may be controlled by scaffold proteins that interact with kinases at different levels in the pathway.

I. Introduction

Mitogen-activated protein kinases (MAPKs) are important mediators for intracellular signaling in cells (Schaeffer and Weber, 1999). Mammalian MAPKs consist of three major groups including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs, also known as stress-activated protein kinases, SAPKs), and p38-MAPKs (Schaeffer and Weber, 1999). All of MAPKs share a common character: they are activated by phosphorylation at a Thr-X-Tyr motif (X is Glu in ERKs, Pro in JNKs, and Gly in p38-MAPKs) in kinase subdomain VIII (Schaeffer and Weber, 1999). The major targets for MAPK kinases are transcription factors that regulate gene expression. MAPKs are involved in signaling induced by various extracellular or intracellular stimuli. Currently, the JNK pathway is one of the known cellular signaling pathways that respond to the most diverse stimuli (Ip and Davis, 1998; Schaeffer and Weber, 1999). JNK is activated by mitogenic signals such as epidermal growth factor (Minden *et al.*, 1994b), lymphocyte activation signals (Su *et al.*, 1994; Sakata *et al.*, 1995; Berberich *et al.*, 1996; Chen *et al.*, 1996a; Chen *et al.*, 1996b), and oncogenic Ras (Derijard *et al.*, 1994). JNK is also activated by pro-inflammatory cytokines (TNF-α and IL-1) (Kyriakis *et al.*, 1994; Sluss *et al.*, 1994), lipopolysaccharide (Hambleton *et al.*, 1996), G protein-coupled receptors (Collins *et al.*, 1996; Coso *et al.*, 1996), shear stress (Li *et al.*, 1996), osmotic shock (Galcheva-Gargova *et al.*, 1994), protein synthesis inhibitors (Kyriakis *et al.*, 1994), and apoptotic stimuli such as growth factor withdrawal (Xia *et al.*, 1995), heat shock (Kyriakis *et al.*, 1994; Zanke *et al.*, 1996), ceramides (Westwick *et al.*, 1995), DNA-damaging chemicals (Saleem *et al.*, 1995; Zanke *et al.*, 1996), UV radiation (Derijard *et al.*, 1994; Chen *et al.*, 1996b; Zanke *et al.*, 1996), and γ radiation (Kharbanda *et al.*, 1995; Chen *et al.*, 1996a; Chen *et al.*, 1996b). The diversity of JNK-activating stimuli imply that mammalian cells may be equipped with multiple upstream regulators that link various cellular signals to the JNK pathway, and the accumulated experimental evidence proves that is the case. To date, the JNK pathway consists of JNKs and various MAP2Ks, MAP3Ks, and MAP4Ks (**Figure 1**). The subtle regulation of

the JNK pathway by its regulators in conjunction with other signaling pathways may allow JNK to regulate a variety of cellular functions. In this review, we will discuss the known components in the JNK pathway and how the emerging mammalian scaffold proteins may control signaling diversity and specificity in this signaling pathway.

II. c-Jun N-terminal kinases

The human JNKs are encoded by three genes *jnk1*, *jnk2*, and *jnk3* (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996). The corresponding genes have also been identified in rats (Kyriakis *et al.*, 1994). JNK3 is preferentially expressed in neuronal tissues, while JNK1 and JNK2 are widely expressed in many tissues. Ten isoforms of JNK, generated by alternative splicing of the transcripts from the three genes, have been identified (Gupta *et al.*, 1996). The protein products of the JNK isoforms have molecular weights of 46 kDa or 55 kDa. The 55 kDa JNK isoforms contain a C-terminal extension, a result of alternative splicing, which distinguishes them from the 46 kDa isoforms (Gupta *et al.*, 1996). No apparent functional differences exist among the 46 kDa and 55 kDa isoforms encoded by the same JNK gene (Gupta *et al.*, 1996). An additional alternative splicing exists in the kinase domains of JNK1 and JNK2, but not in JNK3 (Gupta *et al.*, 1996). The alternative splicing in the kinase domains of JNK1 and JNK2 changes the specificity of interaction between JNKs and their substrates (Gupta *et al.*, 1996), suggesting that JNK isoforms may target different substrates *in vivo*. The JNK binding sites are different from the sites of phosphorylation on the substrates (Kallunki *et al.*, 1996). Deletion of the binding site prevents phosphorylation of the substrate by JNK (Kallunki *et al.*, 1996). However, a substrate lacking a JNK-binding site can also be phosphorylated through association with a protein containing the JNK-binding region (Kallunki *et al.*, 1996).

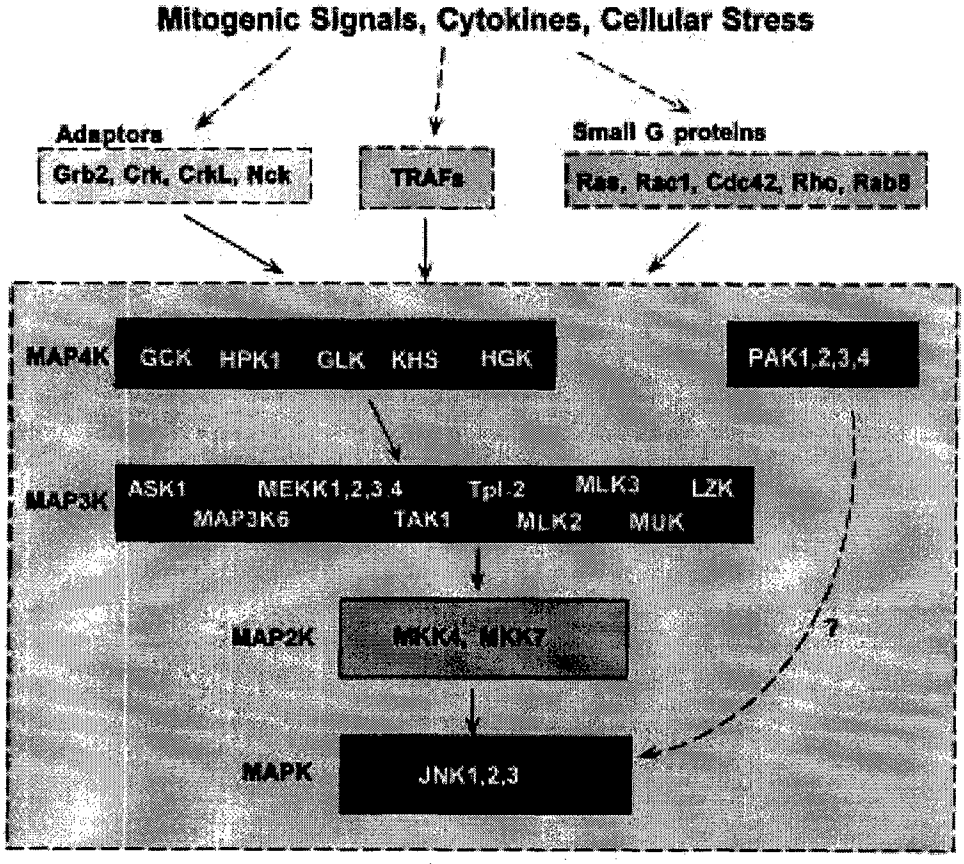

Figure 1. The mammalian JNK signaling pathway. Currently known MAPKs, MAP2Ks, MAP3Ks, and MAP4Ks in the JNK pathway are illustrated schematically. PAKs are capable of activating JNK; however, the direct link between PAKs and the JNK signaling module has not been established. The activation of the JNK pathway is known to be mediated by adaptor molecules, p21 small G proteins, or TNF-receptor-associated factors (TRAFs). The signaling specificity among the components is not presented in this figure.

The known substrates for JNK family members include the transcription factors c-Jun (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Gupta *et al.*, 1996), JunD (Gupta *et al.*, 1996), ATF-2 (Gupta *et al.*, 1995; van

Dam *et al.*, 1995; Whitmarsh *et al.*, 1995), ATFa (Bocco *et al.*, 1996), Elk-1 (Cavigelli *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995), Sap-1a (Janknecht and Hunter, 1997), GABPα, GABPβ (Hoffmeyer *et al.*, 1998), and the tumor suppressor p53 (Milne *et al.*, 1995; Alder *et al.*, 1997). Generally, phosphorylation of these factors by JNK increases their transcriptional activity.

The physiological functions of JNK have been examined by genetic analysis. The *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$ single mutant mice have no global abnormality (Yang *et al.*, 1997b; Dong *et al.*, 1998; Yang *et al.*, 1998). The T cells in *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice preferentially differentiate into Th2 rather than Th1 cells (Dong *et al.*, 1998; Yang *et al.*, 1998). The *jnk1*$^{-/-}$ T cells also hyper-proliferate and exhibit decreased activation-induced apoptosis (Dong *et al.*, 1998). Excitotoxicity-induced apoptosis in the hippocampus is absent in *jnk3*$^{-/-}$ mice in comparison to normal mice (Yang *et al.*, 1997b). The *jnk1/jnk3* and *jnk2/jnk3* deficient mice also develop normally (Kuan *et al.*, 1999); however, *jnk1/jnk2* deficient mice are embryonically lethal and have severe dysregulation of apoptosis in the brain (Kuan *et al.*, 1999). These results indicate that JNK1 and JNK2 may have overlapping functions, and are important in regulation of immune response and embryonic development. JNK3 may have its unique functions in the neuronal tissues. These studies also provide animal models which support the accumulated evidence on the role of JNK in apoptotic signaling in mammalian cells (Ip and Davis, 1998).

III. MAP2Ks in the JNK pathway

The activation of JNK is dependent on the phosphorylation on Thr-183 and Tyr-185. MKK4 (also known as SEK1 or JNKK1) is a physiological activator of JNK (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995). MKK4 phosphorylates and activates JNK *in vitro* and *in vivo* (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995). However, recombinant wild-type JNK proteins are phosphorylated at Tyr, Ser and Thr residues in the presence of recombinant MKK4, whereas a kinase-inactive JNK is phosphorylated predominantly on Tyr (Sanchez *et al.*, 1994). This suggests that recombinant MKK4 does not have apparent dual specificity toward JNK. It is possible that the phosphorylation on Thr-183 is caused by the proline-directed kinase activity of JNK itself, occurring after MKK4-mediated Tyr-phosphorylation. Another possibility is that MKK4 obtains dual-specific kinase activity only after activation by upstream kinases. Two isoforms of MKK4 have been reported through the differential usage of translation initiation sites (Derijard *et al.*, 1995; Lin *et al.*, 1995).

MKK4 has been found to be mutated or deleted in some tumor cells, suggesting that it may be a tumor suppressor gene (Teng *et al.*, 1997; Su *et al.*, 1998). Homologous deletion in *mkk4* genes is embryonically lethal in mice, indicating that MKK4 is essential for embryonic development (Nishina *et al.*, 1997; Yang *et al.*, 1997a). Studies in *mkk4*$^{-/-}$/*rag*$^{-/-}$ chimaeric mice reveal that MKK4 protects thymocytes from apoptosis mediated by CD95 and CD3 (Nishina *et al.*, 1997), and is required for maintenance of a normal peripheral lymphoid compartment but not for lymphocyte development (Swat *et al.*, 1998). *Mkk4*$^{-/-}$ T cells derived from *mkk4*$^{-/-}$/*rag*$^{-/-}$ chimaeric mice are defective in heat shock and anisomycin-induced JNK activation, but normal in osmotic shock-induced JNK activation (Nishina *et al.*, 1997). These results indicate that MKK4 is one but not the only activator of JNK in mammalian cells.

Recently, a novel kinase MKK7 (also named as JNKK2) has been cloned and found to specifically activate JNK, but not p38-MAPK or ERK (Moriguchi *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997). MKK7 is related to MKK4 and belongs to the mammalian MAPK kinase superfamily (Tournier *et al.*, 1997; Yao *et al.*, 1997). MKK7 is also closely related to the *Drosophila* protein kinase hemipterous (HEP) (Tournier *et al.*, 1997; Yao *et al.*, 1997), which is the activator of *Drosophila* JNK (DJNK).

Both MKK4 and MKK7 are widely expressed in human and murine tissue, whereas the relative abundance of each MKK differs among tissues (Tournier *et al.*, 1997; Yao *et al.*, 1997). Both MKK4 and MKK7 mediate signals from the same panel of extracellular stimuli (Wu *et al.*, 1997); however, studies show that they are preferentially activated by different MAP3Ks (Hirai *et al.*, 1998; Merritt *et al.*, 1999; Tournier *et al.*, 1999). Furthermore, the MKK7 gene can encode six isoforms of protein products through alternative splicing of the mRNA transcripts (Tournier *et al.*, 1999). These MKK7 isoforms respond differently to extracellular stimuli and upstream kinases (Tournier *et al.*, 1999). The differential regulation of MKK4 and MKK7 isoforms by their upstream activators needs to be further examined.

IV. MAP3Ks in the JNK pathway

Multiple upstream MAPK/ERK kinase kinases or MAP kinase kinase kinases (MEKK or MAP3Ks) have been reported to activate the JNK pathway via MKK4 and/or MKK7 (**Figure 1**). These MEKK-like kinases include MEKK1-4, ASK1/MAPKKK5, MAPKKK6, TAK1, Tpl-2/Cot, MLK2/MST, MLK3/SPRK/PTK1, MUK/DLK/ZPK, and LZK.

A. MEKKs

MEKK1 is the first identified MAP3K that activates JNK (Minden *et al.*, 1994a; Yan *et al.*, 1994). MEKK1 was cloned on the basis of its homology with the yeast STE11 and Byr2 kinases (Lange-Carter *et al.*, 1993; Xu *et al.*, 1996). To date, four kinases have been cloned and named MEKK1-4 (Lange-

Carter *et al.*, 1993; Blank *et al.*, 1996; Gajewski and Thompson, 1996; Xu *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). The four MEKKs (ranging from 69.5-195 kDa in size) have homologous kinase domains in the C-termini of the proteins; however, their N-terminal domains have little homology. MEKK1 and MEKK4 can interact with GTP-binding proteins Cdc42 and Rac (Fanger *et al.*, 1997; Gerwins *et al.*, 1997). MEKK1 also binds to Ras in a GTP-dependent manner (Russell *et al.*, 1995). All four MEKKs (MEKK1-4) activate the JNK pathway (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Gajewski and Thompson, 1996; Xu *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). Besides the JNK pathway, MEKKs also regulate other cellular signaling pathways. MEKK1, MEKK2, and MEKK3 activate the ERK pathway (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997), and also activate the NF-κB through the IκB kinases (IKKs) (Lee *et al.*, 1997; Zhao and Lee, 1999). MEKK3 and MEKK4 have been shown to activate the p38-MAPK pathway through MKK6 (Takekawa *et al.*, 1997; Deacon and Blank, 1999).

B. TAK1

TGF-β activated kinase 1 (TAK1) was identified by its ability to rescue STE11 mutants in *S cerevisiae* (Yamaguchi *et al.*, 1995). TAK1 is a 579 amino acid protein with the kinase domain in its N-terminus (Yamaguchi *et al.*, 1995). The C-terminal region has no distinct domain structures but interacts with TAK1 binding protein (TAB) 1 and 2 (Yamaguchi *et al.*, 1995; Shibuya *et al.*, 1996). Association of TAK1 and TAB1 enhances the kinase activity of TAK1 (Shibuya *et al.*, 1996). TAK1 is activated by TGF-β (Yamaguchi *et al.*, 1995), interleukin 1 (Ninomiya-Tsuji *et al.*, 1999), ceramide, and UV-C treatments (Shirakable *et al.*, 1997). TAK1 activates JNK and p38, but has no effect on ERK (Yamaguchi *et al.*, 1995; Wang *et al.*, 1997). TAK1 also indirectly activates IKK activity and NF-κB transcriptional activity (Ninomiya-Tsuji *et al.*, 1999). *Xenopus* TAK1 and TAB1 are important in the dorsoventral patterning of early embryos (Shibuya *et al.*, 1998). Ectopic expression of TAK1 induces apoptosis in early *Xenopus* embryos (Shibuya *et al.*, 1998).

C. ASK1/MAPKKK5 and ASK2/MAPKKK6

Apoptosis signal-regulating kinase 1 (ASK1, also named MAPKKK5) was identified by a polymerase chain reaction (PCR)-based strategy (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 consists of 1375 amino acids with a molecular weight about 155 kDa, and the kinase domain of ASK1 is in the middle part of the protein (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 has been shown to activate JNK and p38-MAPK through MKK4 and MKK3 respectively (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 is activated by TNF-α, and dominant-negative ASK1 suppresses TNF-α-induced apoptosis (Ichijo *et al.*, 1997). ASK1 is also activated by Daxx, a Fas-binding protein, and is activated by Fas ligation (Chang *et al.*, 1998). ASK1 has also been shown to be involved in apoptosis induced by oxidative stress (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998), microtubule-interfering agents (Wang *et al.*, 1998a), and genotoxic chemicals (Chen *et al.*, 1999b). Overexpression of ASK1 is capable of inducing apoptosis in transfected cells (Ichijo *et al.*, 1997).

An ASK1-related kinase kinase, MAPKKK6 (murine homologue is ASK2), was identified by yeast two-hybrid screen using ASK1 as a bait (Wang *et al.*, 1998b). MAPKKK6 also interacts with ASK1 when coexpressed in 293 cells (Wang *et al.*, 1998b). Overall, MAPKKK6 is 45% homologous to ASK1, and the kinase domain of MAPKKK6 is 82% identical to that of ASK1. The catalytic domain of MAPKKK6 shares 37, 42, 43, and 42% identity to MEKK1, MEKK2, MEKK3, and MEKK4, respectively (Wang *et al.*, 1998b). In contrast to ASK1, which is a strong JNK and p38-MAPK activator, MAPKKK6 only weakly activates JNK1 but does not activate p38-MAPK or ERK (Wang *et al.*, 1998b).

D. Tpl-2/Cot

Tumor progression locus 2 (Tpl-2) was originally identified as a proto-oncogene that is involved in T lymphomas induced by Moloney murine leukemia virus (Patriotis *et al.*, 1993). Tpl-2 is about 90% identical to the human *cot* gene, which was first identified by its transforming ability (Miyoshi *et al.*, 1991). The Tpl-2 kinase domain shares approximately 30-35% identity to other JNK-activating MAP3Ks; however, the overall similarity between Tpl-2 and other MAP3Ks is low. Expression of Tpl-2 in mammalian cells activates ERK and JNK through the direct phosphorylation of MEK-1 and MKK4, respectively (Salmeron *et al.*, 1996). Tpl-2 has also been shown to participate in CD3 and CD28-induced NF-κB activation through NF-κB-inducing kinase (NIK) and the IKKs (Lin *et al.*, 1999). Tpl-2 also activates the nuclear factor of activated T cells (NF-AT) and induces IL-2 expression in T-cell lines (Tsatsanis *et al.*, 1998).

E. MLKs

The mixed lineage kinase (MLK) family is a group of kinases consisting of MLK1 (Dorow *et al.*, 1993), MLK2/mammalian STE20-like (MST) (Dorow *et al.*, 1993; Katoh *et al.*, 1995), MLK3/src-homology 3 (SH3) domain-containing proline-rich kinase (SPRK)/protein tyrosine kinase 1 (PTK-1) (Rena *et al.*, 1996; Teramoto *et al.*, 1996; Tibbles *et al.*, 1996), MAPK-upstream kinase (MUK)/dual leucine zipper-bearing kinase (DLK)/leucine-zipper protein kinase (ZPK) (Fan *et al.*, 1996; Hirai *et al.*, 1996), and leucine zipper-bearing kinase (LZK) (Sakuma *et al.*, 1997). This group of kinase is characterized by their catalytic domains which show structural features of both tyrosine- and

serine/threonine-specific protein kinase. MLKs contain an SH3 motif at the N-terminus and proline-rich regions at the C-terminus. MLKs also have Leu/Ile-zipper motifs near the C-terminus. These motifs may allow MLKs to dimerize or interact with other molecules. The effect of MLK1 on the MAPK pathways is unknown. MLK2/MST activates the JNK pathway through both MKK4 and MKK7 (Hirai *et al.*, 1998). However, MLK2/MST activates recombinant MKK7 more effectively than recombinant MKK4 (Hirai *et al.*, 1998). MLK2/MST weakly activates p38-MAPK and ERK (Hirai *et al.*, 1997). MLK3 activates the JNK and p38-MAPK pathway via MKK4 and MKK3/6, respectively, but has no effect on the ERK pathway (Tibbles *et al.*, 1996), MUK preferentially activates the JNK pathway (Hirai *et al.*, 1996), and utilizes MKK7 but not MKK4 as a substrate (Merritt *et al.*, 1999). LZK has been shown to activate JNK and induce c-Jun phosphorylation in transfected cells (Sakuma *et al.*, 1997), but its activity toward the other MAPK pathways is unclear. MLK1, 2, 3, and MUK contain potential binding sites (CRIB motifs) for Cdc42 and Rac. MLK2 and MLK3 have been shown to interact with Cdc42 and Rac proteins (Teramoto *et al.*, 1996; Nagata *et al.*, 1998).

V. STE20 related Kinases

The MAP kinase modules in *S. cerevisiae* are controlled by a MAP4K named STE20. Several kinases containing a kinase domain that is homologous to STE20 have recently been identified in mammalian cells (**Figures 2 and 3A**). A phylogenetic analysis on the protein sequences of these mammalian STE20-related kinases reveals that these kinases are divided into several subgroups (**Figure 4**), which roughly correspond to their structures and biochemical properties. The HPK1/GCK subgroup and HGK/NIK have been shown to regulate the JNK pathway through MAP3Ks. Therefore, they could be classified as MAP4Ks in mammalian cells.

A. PAK subgroup

p21-activated kinases (PAK1-4) are characterized by their ability to bind to the Ras-related small G-proteins, Rac1 and Cdc42, through their CRIB domains (Bagrodia *et al.*, 1995b; Martin *et al.*, 1995; Abo *et al.*, 1998) (**Figure 2**). The binding of PAKs to GTP-bound Rac1 or Cdc42 results in the autophosphorylation and activation of the kinase (Manser *et al.*, 1994; Bagrodia *et al.*, 1995b; Martin *et al.*, 1995; Teo *et al.*, 1995). Rac1 and Cdc42, as well as their direct guanine nucleotide exchange factors (GEF), Ost and Dbl, respectively, were shown to stimulate the JNK pathway via MEKK1 (Bagrodia *et al.*, 1995a; Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Brown *et al.*, 1996). However, PAKs do not behave as MAP4Ks in the JNK pathway, since PAKs have no (Yablonski *et al.*, 1998; Zhou *et al.*, 1998) or only modest (Bagrodia *et al.*, 1995a; Polverino *et al.*, 1995; Frost *et al.*, 1996) effect on JNK activation. In addition, the direct interaction between PAKs and MAP3Ks has not been shown. It is found that PAK binding to Rac1 is dispensible for Rac1-induced activation of JNK1 (Westwick *et al.*, 1997). Since Cdc42 and Rac can directly interact with MEKK1, MEKK4, MLK2 and MLK3, PAKs may not be the direct link between small G proteins and the JNK kinase cascade. Neutrophil PAK1 and PAK2 have been shown to phosphorylate the $p47^{phox}$ subunit of NADPH oxidase in response to a chemotactic peptide fMetLeuPhe (Knaus *et al.*, 1995). Recombinant PAK also phosphorylate $p67^{phox}$ subunit of NADPH oxidase (Ahmed *et al.*, 1998). Therefore, it is possible that PAKs indirectly regulate JNK activation by inducing chronic oxidative stress through NADPH oxidase in cells.

B. GCK/HPK1 subgroup

The second subgroup of STE20-related kinases includes germinal center kinase (GCK), hematopoietic progenitor kinase 1 (HPK1), kinase homologous to STE20 (KHS)/GCK related kinase (GCKR), and GCK-like kinase (GLK). These kinases are around 96-97 kDa, and share a similar structural configuration. They have the STE20-like catalytic domain in their N-terminus, at least 2 proline-rich binding domains, which can bind to the Src homology 3 (SH3) domain, in their middle region. A domain distantly related to part of murine citron protein (citron homology domain, CNH domain) is located in the C-terminus of these kinases (**Figures 2 and 3**). This subgroup of STE20-related kinases have been shown to be strong and specific JNK activators.

Germinal center kinase (GCK) was first found to be expressed in B lymphocytes residing in the germinal center region of lymphoid follicles (Katz *et al.*, 1994), but later found to be ubiquitously expressed in many tissues. It is a potent and specific activator of JNK but not of ERK1 or p38-MAPK (Pombo *et al.*, 1995). GCK-mediated JNK activation was blocked by a dominant-negative MKK4/SEK construct which indicates that GCK also activates MKK4/SEK (Pombo *et al.*, 1995); however, a direct interaction between GCK and MKK4 has not been detected. GCK has been shown to interact with TNF receptor-associated factor-2 (TRAF2) and with MEKK1 (Yuasa *et al.*, 1998). Therefore, GCK may link the TNF receptor complex to the JNK pathway through MEKK1. GCK interacts with small G protein Rab8 (Ren *et al.*, 1996); however, the biological significance of this interaction is unclear.

Hematopoietic progenitor kinase 1 (HPK1) is preferentially expressed in hematopoietic cells, especially in lymphocytes (Hu *et al.*, 1996; Kiefer *et al.*, 1996). HPK1 does not contain CRIB motif and does not interact with Rac1 or Cdc42 (Hu *et al.*, 1996). HPK1 contains four proline-rich domains (putative SH3 domain-binding sites) (**Figure 2**). HPK1 has been shown

to interact with the adaptor molecules Grb2, Crk, CrkL and Nck (Anafi *et al.*, 1997; Oehrl *et al.*, 1998; Ling *et al.*, 1999). These adaptor proteins are involved in signaling induced by receptor-linked tyrosine kinases through their SH2 domains, which bind to phosphorylated tyrosine residues. Fas receptor signaling results in the caspase-mediated-cleavage of HPK1 at aspartic acid residue 385, which leads to an increase of HPK1 kinase activity and a decrease in its binding to Crk and Grb2 (Chen *et al.*, 1999a). Several pieces of evidence show that HPK1 is regulated by tyrosine kinases. HPK1 is activated by tyrosine phosphatase inhibitors and is tyrosine-phosphorylated after epidermal growth factor stimulation and T-cell receptor ligation (Anafi *et al.*, 1997; Ling *et al.*, 1999). The interaction between HPK1 and adaptor proteins may recruit HPK1 to surface receptor-tyrosine kinase complexes. HPK1 preferentially activates JNK but not ERK or p38-MAPK (Hu *et al.*, 1996; Kiefer *et al.*, 1996). HPK1 interacts with MEKK1 *in vivo* and directly phosphorylates its regulatory region *in vitro* (Hu *et al.*, 1996). HPK1 also interacts with MLK3 and TAK1 (Kiefer *et al.*, 1996; Zhou *et al.*, 1999). HPK1 is upstream of TAK1 in TGF-β induced JNK activation, and the interaction between HPK1 and TAK1 is enhanced by TGF-β treatment (Zhou *et al.*, 1999).

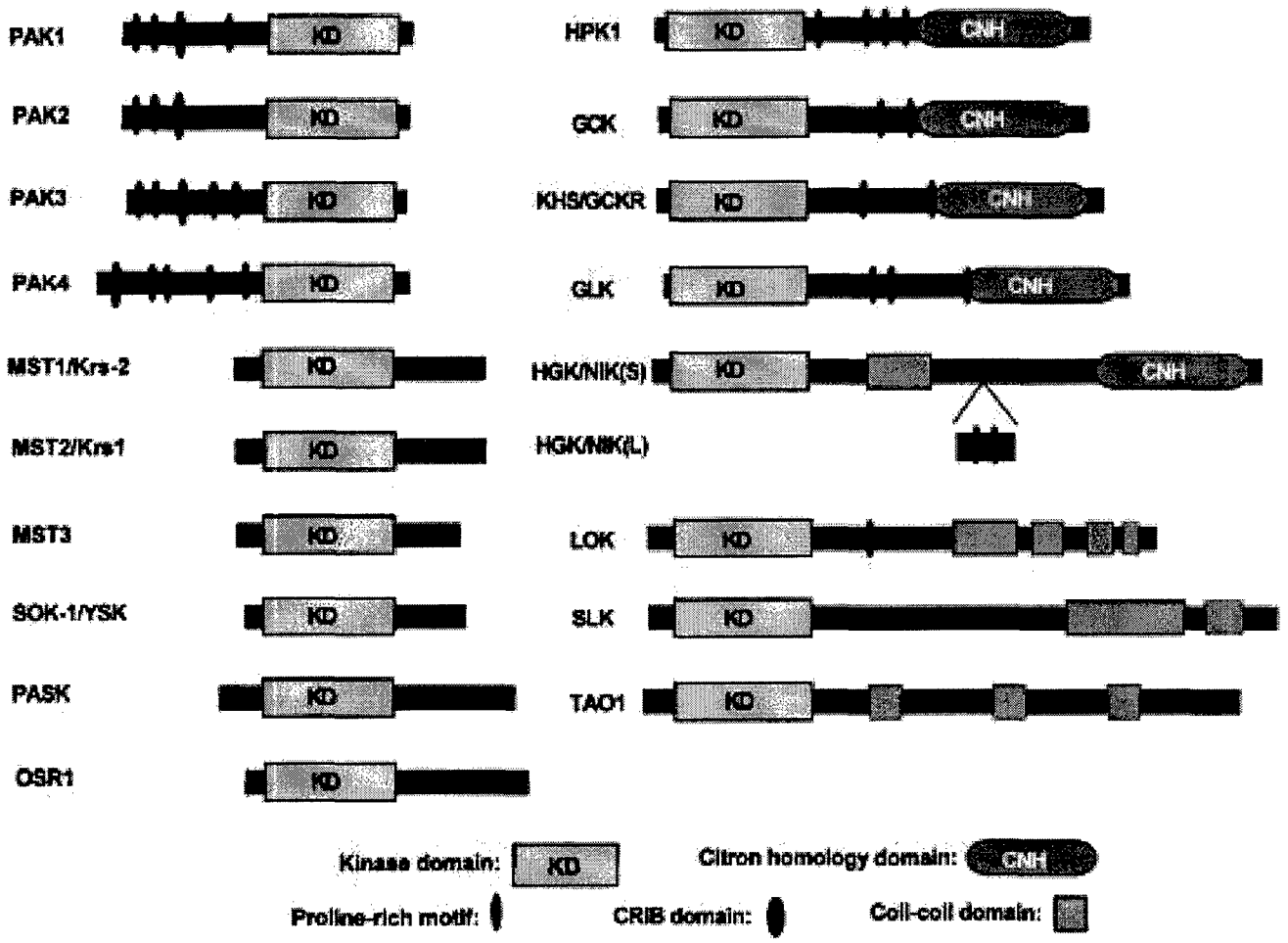

Figure 2. Structures of mammalian STE20-related kinases. The protein sequences of the STE20-related kinases were analyzed using the Web-based SMART program (simple modular architecture research tool, EMBL). Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

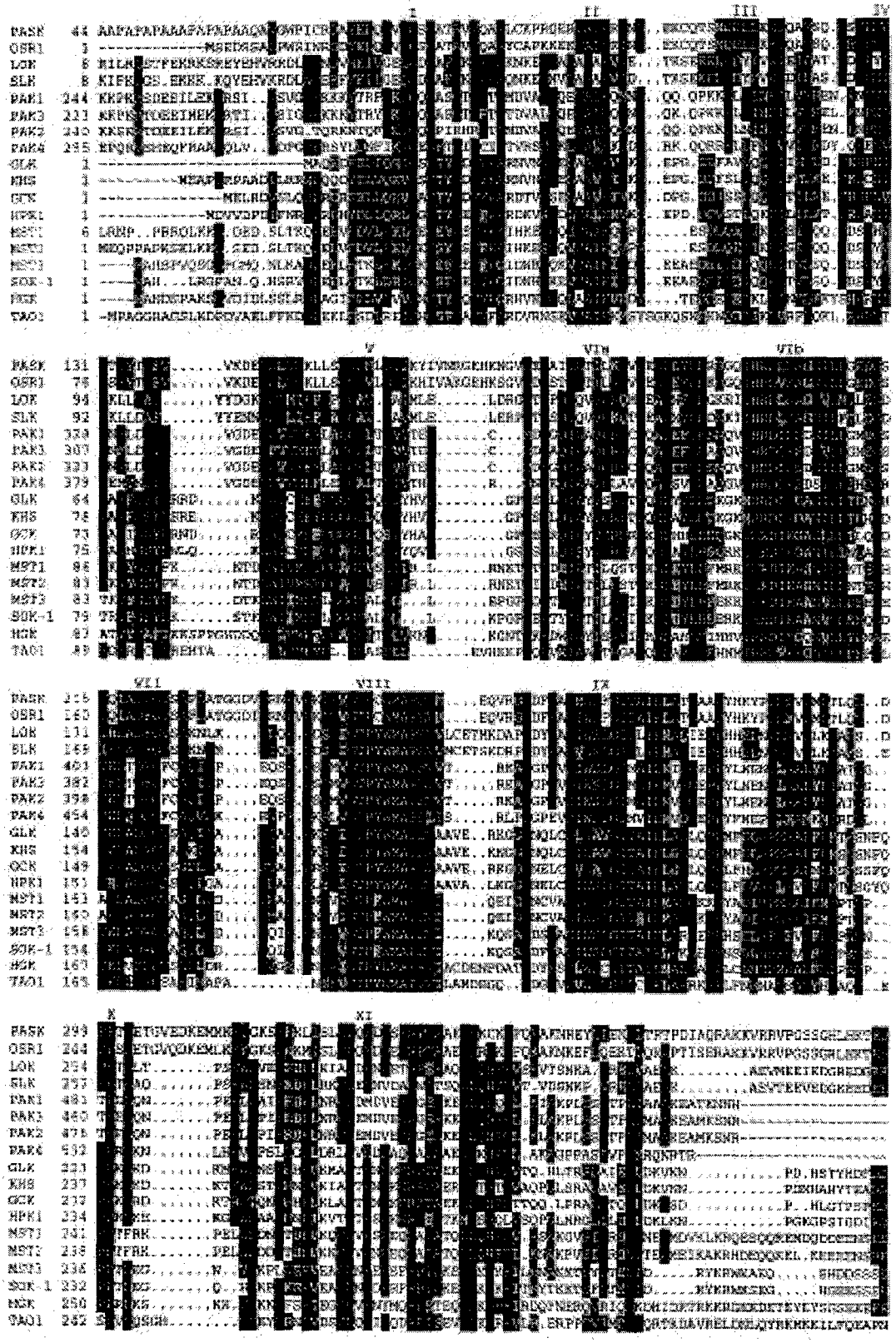

Figure 3. Sequence comparison among mammalian STE20-related kinases. (**A**) Alignment among the kinase domains of STE20-related kinases. The percentages of identity vary from 95% to 36%. Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

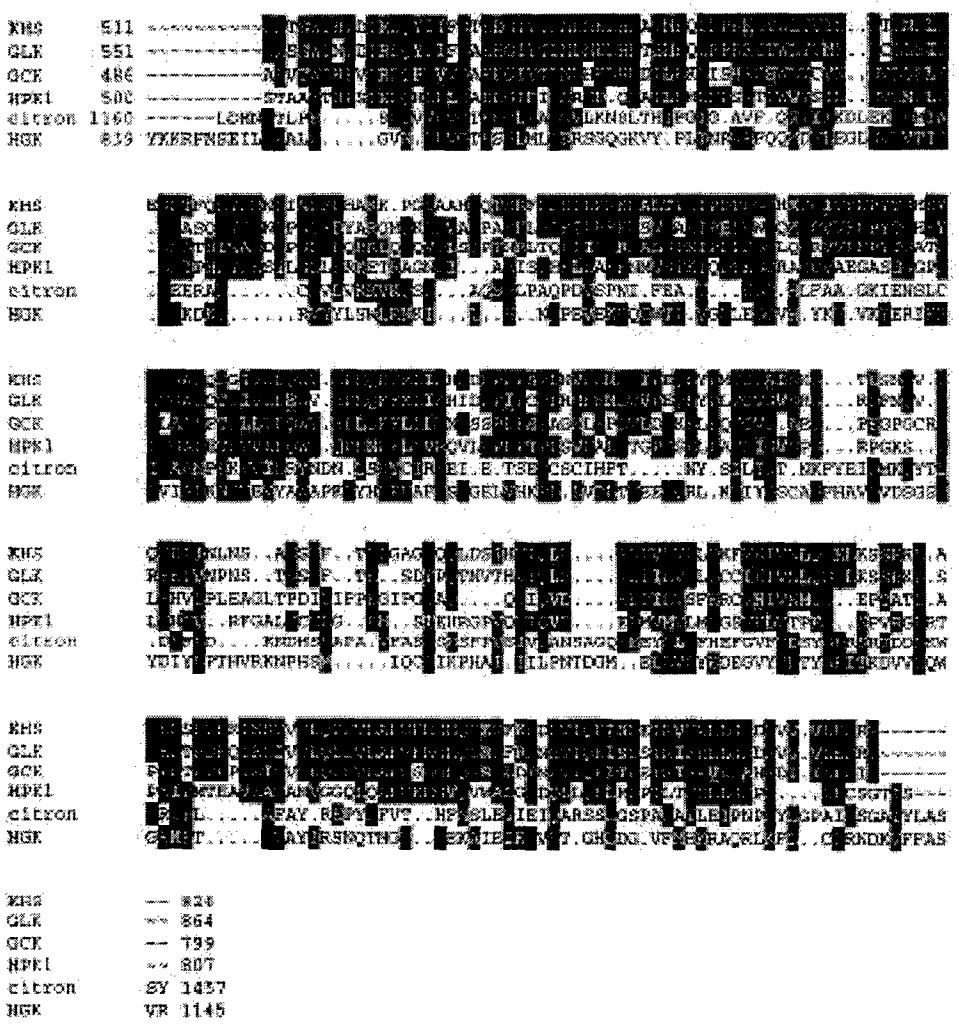

Figure 3 (Continued). **(B)** Alignment of citron homology domains of HPK1, GCK, KHS, GLK, HGK and murine citron protein. The sequences were analyzed using the PILEUP program (version 7.2; Wisconsin Genetics Computer Group). The output was processed using the BoxShade 3.21 program. Identical residues are shown in black shade, and conserved residues are shown in gray shade. Gaps introduced into the sequences to optimize the alignment are illustrated with dots.

KHS/GCKR is a kinase closely related to GCK and HPK1 (Shi and Kehrl, 1997; Tung and Blenis, 1997). KHS/GCKR also preferentially activates JNK but not p38-MAPK and ERK (Shi and Kehrl, 1997; Tung and Blenis, 1997). KHS/GCKR is activated by TNF-α and UV irradiation (Shi and Kehrl, 1997). A KHS/GCKR dominant-negative mutant or antisense suppresses TNF-α, TRAF2, and UV-induced JNK activation (Shi and Kehrl, 1997). KHS/GCKR also physically interacts with TRAF2 (Shi and Kehrl, 1997). KHS/GCKR interacts with the SH3 domains of Crk and CrkL, but not with the SH3 domains of Grb2 or Nck, through its proline rich domains (Oehrl *et al.*, 1998). KHS/GCKR is constitutively active in chronic myeloid leukemia (CML) cells and interacts with oncoprotein Bcr-Abl (Shi *et al.*, 1999). KHS/GCKR is activated by Bcr-Abl in a Ras-dependent manner (Shi *et al.*, 1999). A dominant-negative KHS/GCKR blocks Bcr-Abl-induced JNK activation (Shi *et al.*, 1999).

GLK is widely expressed in many tissues (Diener *et al.*, 1997). GLK preferentially activates JNK, but not p38-MAPK or ERK, when co-expressed in mammalian cells (Diener *et al.*, 1997). GLK-induced JNK activation is blocked by a dominant-negative mutant of MKK4 or MEKK1 (Diener *et al.*, 1997). GLK phosphorylates recombinant MEKK1 (Diener *et al.*, 1997). These data suggest that GLK regulates the JNK pathway through MEKK1 and MKK4. To date, GLK is known to be regulated by UV irradiation and TNF-α (Diener *et al.*, 1997). Since GLK also contains proline-rich motifs, like other kinases in this family (**Figure 2**), it may be regulated through interaction with SH3 domain-containing molecules.

C. *HGK, LOK, SLK, and TAO1*

Human HPK/GCK like kinase (HGK) is a 133.4 kDa protein (Yao *et al.*, 1999). Its murine counterpart, Nck-interacting kinase (NIK), is 98% identical to HGK, except for an insertion containing two proline-rich motifs in the middle region of the kinase (Su *et al.*, 1997) (**Figure 2**). A longer form of human HGK that contains these proline-rich motifs was also detected in brain tissue by RT-PCR (Yao *et al.*, 1999). However, the short form of HGK appears to be the predominant form in other human tissues including liver, skeletal muscle and placenta (Yao *et al.*, 1999). Murine HGK/NIK activates JNK and MKK4 when co-expressed in cells, and interacts with MEKK1 (Su *et al.*, 1997). Murine HGK/NIK strongly interacts with the SH3 domain of Nck, but not with other molecules that contain SH3 domains, such as Grb2 and phospholipase C-γ (Su *et al.*, 1997). Therefore, it may link Nck-mediated signals to the JNK pathway through MEKK1 and MKK4. Human HGK/NIK-induced JNK activation can be blocked by a dominant-negative mutant of MKK4, MKK7, or TAK1, but not by a dominant-negative MEKK1 mutant (Yao *et al.*, 1999). The difference between human and murine HGK/NIK (long versus short forms) is intriguing and needs to be further examined. Although HGK/NIK contains a citron-homology (CNH) domain (**Figure 2**), this domain shares low homology to the CNH domains in HPK1, GCK, KHS, and GLK (**Figure 3B**). HGK/NIK also contains a coiled-coil domain that is not found in any other kinase of the GCK/HPK1 subgroup (**Figure 2**). A phylogenetic analysis reveals that HGK does not belong to the HPK1/GCK subgroup (**Figure 4**).

Lymphocyte-oriented kinase (LOK) is a 130 kDa kinase which contains a STE20-like kinase domain in its N-terminus followed by a proline-rich region that is a potential SH3 domain binding site, and a long coiled-coil structure at its C-terminus (Kuramochi *et al.*, 1997; Kuramochi *et al.*, 1999) (**Figure 2**). LOK, unlike HPK1/GCK subgroup, only weakly activates the JNK and p38-MAPK pathways by itself (Kuramochi *et al.*, 1997). LOK may have a specific function in lymphocytes; however, the physiological stimuli and downstream effectors for LOK are unknown (Kuramochi *et al.*, 1997).

Thousand and one amino acid protein kinase 1 (TAO1) was identified by a PCR-based method from a rat brain cDNA library (Hutchison *et al.*, 1998). TAO1 is preferentially expressed in brain and testis (Hutchison *et al.*, 1998). TAO1 contains a kinase domain which shares 40-50 % homology with other STE20-related kinases, and a C-terminal regulatory domain which contains coiled-coil structures (Hutchison *et al.*, 1998) (**Figure 2**). TAO1 activates recombinant MKK4, MKK3, and MKK6 *in vitro*; however, it preferentially interacts with MKK3 and only activates MKK3 when co-expressed in mammalian cells (Hutchison *et al.*, 1998).

STE20-like kinase (SLK) is a 141 kDa kinase (Itoh *et al.*, 1997; Pytowski *et al.*, 1998). The N-terminal catalytic domain of SLK shares 70% identity with LOK's kinase domain, and 40-45% identity with the kinase domains of other STE20-related kinases. The C-terminus of SLK shares 40% homology with LOK, 20% homology with HGK and TAO1, and no homology with other STE20-related kinases. The specificity of SLK toward the known MAPK pathways is unknown.

D. MST/SOK subgroup

This subgroup includes mammalian STE20 like 1 (MST1)/kinase regulated by stress 2 (Krs2) (Creasy and Chernoff, 1995a; Taylor *et al.*, 1996), MST2/Krs1 (Creasy and Chernoff, 1995b; Taylor *et al.*, 1996), MST3 (Schinkmann and Blenis, 1997), STE20/oxidant stress response kinase-1 (SOK-1)/yeast Sps1/Ste20-related kinase 1 (YSK1) (Pombo *et al.*, 1996; Osada *et al.*, 1997), and proline-alanine-rich Ste20-related kinase (PASK) (Ushiro *et al.*, 1998). These kinases are approximately 45-55 kDa and share 55-90% identity in their STE20-like kinase domain. These kinases have not been shown to activate any of the MAPK pathways, however, their abilities to affect MAPK signaling in the presence of MAPK-inducing agents have not been tested. MST1/Krs2 and MST2/Krs1 are activated by extreme heat shock (55°C), arsenite, okadaic acid, and staurosporine (Taylor *et al.*, 1996). MST1/Krs2 is also activated by *in vitro* incubation with purified PP2A (Creasy and Chernoff, 1995a). SOK-1/YSK1, as its name indicates, is activated by oxidative stress such as H_2O_2 (Pombo *et al.*, 1996). The stimuli that activate MST3 are unknown.

VI. Scaffold Proteins

Since JNK is regulated by such diverse upstream pathways, the mechanism by which cells ensure signaling specificity is intriguing. In *S. Cerevisiae*, the MAPK kinase pathways are coordinated by scaffold proteins (Herskowitz, 1995; Whitmarsh and Davis, 1998). The Ste5p protein binds the components of the MAPK module that control mating (Herskowitz, 1995; Whitmarsh and Davis, 1998). Ste5p interacts with the MAP3K Ste11, the MAP2K Ste7, and the MAPKs Fus3p and Kss1p through different regions of the protein. Pbs2p is a scaffold protein that coordinates an osmoregulatory MAPK pathway (Herskowitz, 1995; Whitmarsh and Davis, 1998). In contrast with Ste5p, Pbs2p is not only a scaffold, but also a protein kinase component (MAP2K) of this MAPK pathway. In mammalian cells, the understanding of scaffold proteins is limited.

Recent studies indicate that mammalian cells do contain proteins which serve as scaffolds. One such protein which facilitates signaling of the JNK pathway has been identified. JNK-interacting protein 1 (JIP1) was isolated by a two-hybrid screen for proteins that bind to JNK. JIP1 preferentially binds to JNK, but not to ERK or p38-MAPK (Whitmarsh *et al.*,

1998). JIP1 was first characterized as a JNK inhibitor, because it inhibits the nuclear translocation of JNK and suppresses JNK-mediated functions including transformation and apoptosis (Dickens *et al.*, 1997). However, further characterization of JIP1 has shown that JIP1 interacts with multiple components of the JNK cascade (Whitmarsh *et al.*, 1998). JIP1 binds to MKK7 but not MKK4, which activates both JNK and p38-MAPK. JIP1 also selectively interacts with the MLK family of MAP3K. MKK7 and MLK bind to regions on JIP1 that are distinct from the JNK binding site (Whitmarsh *et al.*, 1998). HPK1 also interacts with JIP1; however, whether this interaction is direct or mediated through MLK family members is uncertain (Whitmarsh *et al.*, 1998). These results strongly suggest that JIP1 serves as a scaffold protein in mammalian cells like Ste5p in yeast cells.

Mammalian MAP kinase pathways are much more complicated than those of yeast. It is unknown whether each kinase cascade is coordinated by a specific scaffold protein. Several pieces of evidence suggest that some mammalian kinases may serve as scaffold proteins, similar to Pbs2p in yeast. For example, MEKK1 is capable of interacting with JNK (Xu and Cobb, 1997), MKK4 (Su *et al.*, 1997), NIK (murine HGK) (Su *et al.*, 1997), and HPK1 (Hu *et al.*, 1996) through different regions of the MEKK1 protein. These properties enable MEKK1 to serve as a scaffold protein. However, whether MEKK1 can bind to all of these kinases simultaneously and the contribution of this binding to signaling specificity is unknown. Several pieces of evidence suggest that certain stimuli may use distinct kinase cascades to activate JNK through signal-specific scaffold proteins. For example, TAK1-induced JNK activation can be blocked by a dominant-negative mutant of MKK4 or MKK7, suggesting that both MKK4 and MKK7 have the potential to mediate TAK1-induced JNK activation (Zhou *et al.*, 1999). However, TGF-β-induced JNK activation, which is mediated by TAK1, is blocked by the dominant-negative mutant of MKK4, but not by a MKK7 mutant (Zhou *et al.*, 1999). These data suggest that TGF-β signaling specifically uses the TAK1-MKK4-JNK cascade, but not the TAK1-MKK7-JNK cascade, implying a signal-specific scaffold may be involved.

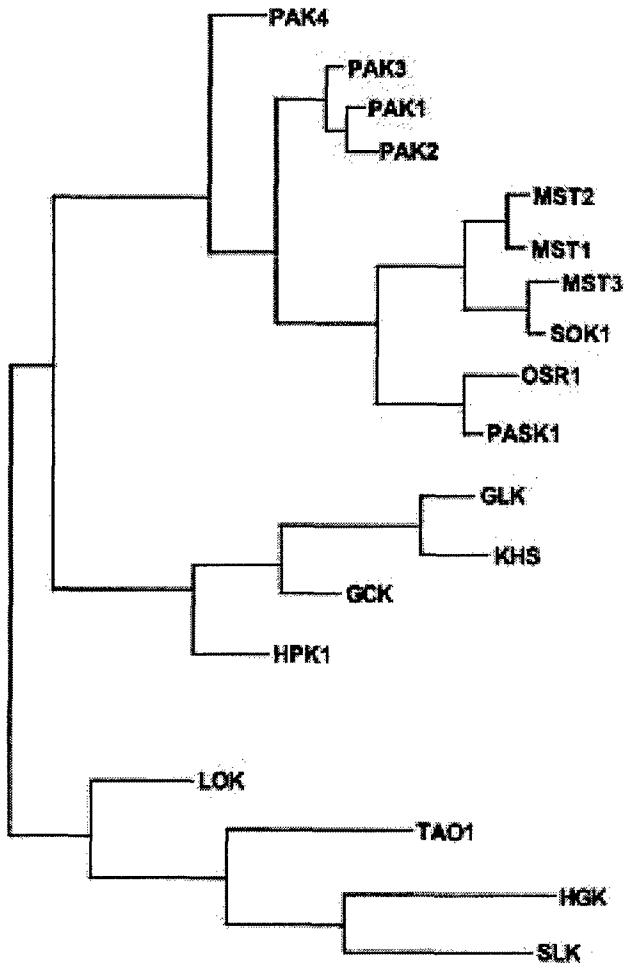

Figure 4. Relationship between members of mammalian STE20-related kinases. The phylogenetic analysis was performed using the multiple alignment server provided by the DNA DataBank of Japan (malign@nig.ac.jp). Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

VII. Conclusion

The c-Jun N-terminal kinase pathway is activated by a variety of extracellular and intracellular stimuli. The biochemical mechanisms by which these stimuli converge upon and regulate the JNK pathway are intriguing. The discovery of multiple upstream JNK regulators, especially at the MAP3K and MAP4K levels, suggests that these kinases may connect the JNK signaling module to the upstream signals. However, the involvement and requirement of these kinases in JNK activation by specific stimuli remains unclear. To date, the studies on these kinases rely upon transient transfection assay, or on the examination of the activation of endogenous kinases by certain stimuli. The dominant-negative kinase mutants are useful to determine the possible involvement of these kinases in response to a specific stimulus; however, the establishment of genetically deficient animals or cell lines would be extremely critical to elucidate the biochemical and physiological importance of these JNK activators. In addition, revealing the further upstream regulators that link MAP3Ks and MAP4Ks to stimuli will be important.

JNK isoforms appear to have different substrate specificity. MKK4 and MKK7 also seem to have different substrate specificity, and are differentially regulated by upstream activators. The expanding molecules in the MAP3K and MAP4K levels further create complexity and diversity in the signaling specificity. The identification of scaffold-like proteins, such as JIP1, in mammalian cells indicates that the signaling molecules in the MAPK pathways may form complexes through interactions with scaffold proteins. Different complexes, with distinct components, may mediate different upstream signals and regulate distinct downstream effectors. Although the emergence of the scaffold model provides an explanation for the control of signaling specificity, many questions arise. Does the formation of these complexes occur before or after the stimulation? Is the specificity of these scaffold proteins stringent, or is it relatively flexible? Are these signaling complexes stable, or can the components in different complexes be exchanged rather freely? The answers to these questions will greatly enhance the understanding of cellular signaling.

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Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate

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Oxidative stress activates the c-Jun N-terminal kinase (JNK) pathway. However, the exact mechanisms by which reactive oxygen species (ROS) activate JNK are unclear. We found that the ability of hydrogen peroxide (H_2O_2) to induce JNK activation varied in different cell types. Pyrrolidine dithiocarbamate (PDTC), a presumed antioxidant, induced JNK activation on its own and enhanced JNK activation by H_2O_2 in many cell types, including Jurkat, HEK293, and LNCaP and Tsu-Pr1 prostate cancer cells. The activation of JNK by PDTC, in the presence or absence of exogenous H_2O_2, was dependent on its chelating ability to metal ions, most likely copper ions. Despite the strong JNK-activating ability, H_2O_2 plus PDTC did not induce significant activation of the upstream kinases, SEK1/MKK4 and MKK7. However, the JNK inactivation rate was slower in cells treated with H_2O_2 plus PDTC compared with the rate in cells treated with ultraviolet C (UV-C). Treatment of H_2O_2 plus PDTC significantly decreased the expression levels of a JNK phosphatase, M3/6 (also named hVH-5), but not the levels of other phosphatases (PP2A and PP4). In contrast, UV-C irradiation did not cause the down-regulation of M3/6. These results suggest that JNK activation by H_2O_2 plus PDTC resulted from the down-regulation of JNK phosphatases. Our data also reveal a necessity to carefully evaluate the pharmacological and biochemical properties of PDTC. *Oncogene* (2001) **20,** 367–374.

Keywords: JNK; phosphatase; oxidation; metal ions

Introduction

Oxygen and transition metal ions are required for normal cellular metabolism. However, the existence of oxygen and metal ions in cells is potentially dangerous because of the unwanted toxicity, partially derived from the production of reactive oxygen species (ROS) (reviewed in Halliwell and Gutteridge, 1990; Jacobson, 1996). Oxygen (O_2) can receive a free electron and form superoxide anions ($O_2^{\bullet}$). Superoxide anions can induce the production of hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Hydroxyl radicals are highly reactive, and can cause damage in the proximity of where they are generated in a cellular environment (Jacobson, 1996). In mammalian cells, superoxide anions are continuously generated by NADPH oxidase, monoamine oxidase and, mostly, by the electron transport system in mitochondria (Hauptmann and Cadenas, 1997; Jacobson, 1996). The superoxide anions are converted by superoxide dismutase (SOD) into relatively inert H_2O_2. H_2O_2 can be reduced to H_2O and O_2 by catalase or by glutathione (GSH) peroxidase. H_2O_2 can also be decomposed into hydroxyl radicals through nonenzymatic pathways by transition metal ions (Cu^+ or Fe^{2+}). Therefore, the tight coupling of SOD, catalase, and peroxidase is important for reducing the generation of hydroxyl radicals inside the cells (Hauptmann and Cadenas, 1997). Increases in ROS production, defects in the ROS-removing enzymatic system, or decreases in antioxidant levels may cause serious damage to cells (Jacobson, 1996). The necessity of maintaining the redox balance is underscored by the evidence that many apoptotic stimuli induce oxidative stresses directly or indirectly (reviewed in Fuchs *et al.*, 1997; Jacobson, 1996).

JNK (also called stress-activated protein kinase, SAPK) family members belong to the mitogen-activated protein kinase (MAPK) superfamily which also includes extracellular signal-regulated kinases (ERKs) and the p38-MAPK family (reviewed in Chen and Tan, 2000; Ip and Davis, 1998). The JNK pathway responds to many diverse stimuli including mitogens, fluid shearing, pro-inflammatory cytokines, and environmental stresses including various apoptotic stimuli (reviewed in Chen and Tan, 2000; Ip and Davis, 1998; Schaeffer and Weber, 1999). The human JNKs are encoded by three genes *jnk1*, *jnk2*, and *jnk3* (Derijard *et al.*, 1994; Gupta *et al.*, 1996; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). The corresponding murine genes have also been identified (Kyriakis *et al.*, 1994). JNK1 and JNK2 are ubiquitously expressed in many tissues, while JNK3 is predominantly expressed in neuronal tissues. Ten isoforms of JNK are generated by alternative splicing of the transcripts from the three genes (Gupta *et al.*, 1996). The known JNK pathway consists of JNKs and various MAPK kinases (MAP2Ks), MAPK kinase kinases (MAP3Ks), and MAPK kinase kinase kinases (MAP4Ks), which may

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mediate signals induced by distinct stimuli (reviewed in Chen and Tan, 1999; Ip and Davis, 1998). Activated JNK can be dephosphorylated and inactivated by a number of dual-specificity phosphatases (reviewed in Camps *et al.*, 1999).

The JNK pathway is activated by oxidative stress; however, the mechanism is unclear. Recently, several enzymes involved in redox metabolism were found to interact with molecules in the JNK pathway. Thioredoxin interacts with a JNK activator, apoptosis-activating kinase 1 (ASK1) (Saitoh *et al.*, 1998), and glutathione S-transferase Pi (GSTp) interacts with JNK (Adler *et al.*, 1999). Thioredoxin inhibits ASK1 activity, and treatment of H_2O_2 leads to the dissociation of thioredoxin-ASK1 complex and ASK1 activation (Saitoh *et al.*, 1998). The interaction between GSTp and JNK, which is dissociated under oxidative stress, prevents JNK phosphorylation of c-Jun (Adler *et al.*, 1999). This evidence suggests that the JNK pathway responds to oxidative stress using the associated redox enzymes as sensors. In this study we used H_2O_2 and PDTC as probes to examine the effects of oxidative stress and transition metal ions on JNK signaling. Our results suggest that JNK is activated by oxidative stress and by metal toxicity through the down-regulation of the dual-specificity phosphatases, which negatively regulate JNK. Our data also reveal a necessity to re-examine the pharmacological and biochemical properties of PDTC.

Results

H_2O_2 and PDTC synergistically induce JNK activation

Previously, we and others showed that oxidative stress participates in JNK activation by various stimuli (Chen *et al.*, 1998b; Luo *et al.*, 1998; Park *et al.*, 1996; Wilhelm *et al.*, 1997). However, it has also been shown that antioxidant treatments caused JNK activation (del Arco *et al.*, 1996) which contradicts the notion that oxidative stress activates JNK. To examine the controversial effect of cellular reduction-oxidation (redox) status on the JNK pathway, we tested the effect of H_2O_2 on JNK activation. We found that although exogenous H_2O_2 (100 μM) activated JNK in Jurkat T cells, it failed to induce JNK activation in HEK293, LNCaP, and Tsu-Pr1 cells (Figure 1a–d). In contrast, PDTC, a presumed antioxidant, activated JNK in HEK293, LNCaP, and Tsu-Pr1 cells but not in Jurkat cells. PDTC and H_2O_2 have a synergistic effect on JNK activation in all of the cell lines tested (Figure 1a–d). The oxidative property of H_2O_2 is undisputed; therefore, the synergistic JNK-activating effect between H_2O_2 and PDTC is contradictory to the presumed anti-oxidative property of PDTC.

Extracellular metal ions are required for PDTC-induced JNK activation

In addition to its reported anti-oxidative property, PDTC is capable of binding to various transition metal ions including zinc, iron, copper and manganese (Gilman *et al.*, 1990). PDTC was also reported to increase intracellular copper and zinc ions by transporting extracellular ions across the cell membrane (Kim *et al.*, 1999; Nobel *et al.*, 1995; Verhaegh *et al.*, 1997). Transition metal ions, such as ferrous (Fe^{2+}) or cuprous (Cu^{+}), are known to generate hydroxyl radicals ($^{\bullet}OH$) by decomposing H_2O_2 (Halliwell and Gutteridge, 1990). Therefore, PDTC may increase the intracellular metal ions and, subsequently, the production of hydroxyl radicals in the presence of H_2O_2.

To determine whether transition metal ions are required for PDTC-induced JNK activation, we removed serum, the major source of transition metal ions, from the experimental condition. H_2O_2, PDTC, or H_2O_2 plus PDTC was unable to induce JNK activation in HEK293 cells in the absence of serum (Figure 2a), suggesting that some components in serum are required for JNK activation. Because cupric ions (Cu^{2+}) can be easily reduced to cuprous ions (Cu^{+}) in physiological conditions, and can react with H_2O_2 to generate hydroxyl radicals, we tested whether the addition of Cu^{2+} in the serum-free condition can restore PDTC's JNK-activating ability. PDTC, in the presence or absence of exogenous H_2O_2, did activate JNK activation when $CuSO_4$ was included in the serum-free medium (Figure 2b). PDTC caused weak JNK activation in the presence of zinc ions; however, no synergistic effect between PDTC and H_2O_2 on JNK activation was detected in the presence of zinc in a serum-free condition (Figure 2b). Other metal ions tested, including ferrous (Fe^{2+}) and manganese (Mn^{2+}) ions, were unable to induce significant JNK activation in the presence of PDTC in a serum-free condition (data not shown).

To examine if copper ions can fully replace the requirement of serum in JNK activation by PDTC and H_2O_2, we compared the levels of JNK activation by PDTC plus H_2O_2 in cells treated with serum or with copper ions in the absence of serum. The normal range of the copper ions in serum is 10–30 μM (Lentner, 1984). We found that the magnitudes of JNK activation were comparable for cells treated with 10% serum and cells treated with 3 μM of copper ions in the absence of serum (Figure 2c). A stronger JNK activation was achieved with a higher concentration (30 μM) of copper ions (Figure 2c). Taken together, these results support an important role of extracellular copper ions in PDTC-mediated JNK activation.

PDTC-induced JNK activation is suppressed by a nonpermeable cuprous ion chelator

To test whether the translocation of extracellular copper ions into cells is required for JNK activation by PDTC, we incubated the HEK293 cells with a nonpermeable cuprous ion (Cu^{+}) chelator (Nobel *et al.*, 1995; Verhaegh *et al.*, 1997), bathocuproinedisulfonic acid (BCS), before the treatments. Presumably, BCS can compete with PDTC for extracellular copper ions and reduce the influx of copper ions mediated by

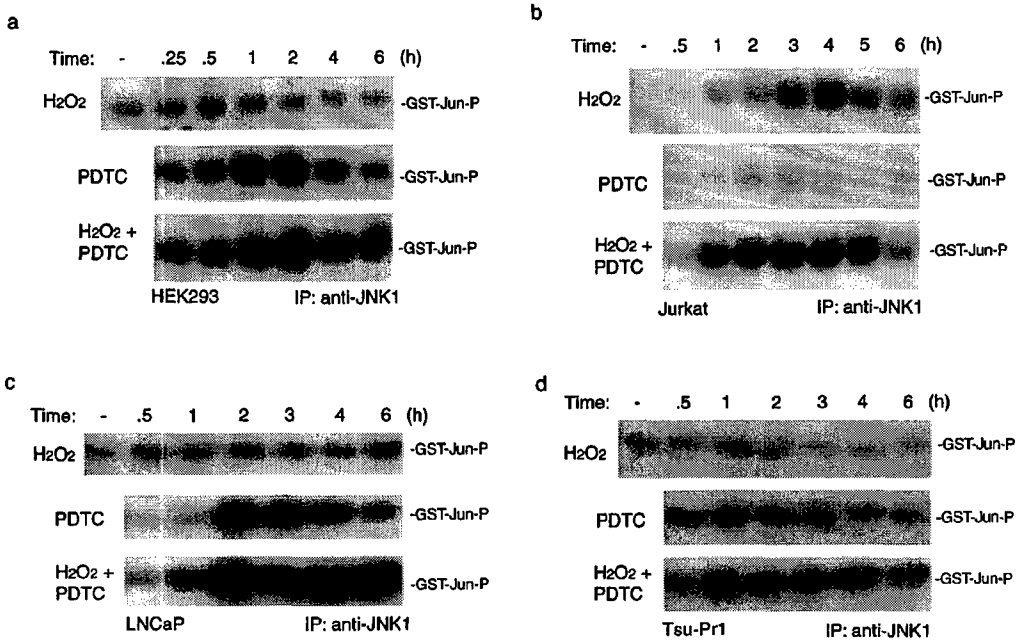

Figure 1 Activation of JNK by H_2O_2 and/or PDTC. HEK293 (**a**), Jurkat (**b**), LNCaP (**c**), and Tsu-Pr1 (**d**) cells were treated with H_2O_2 (100 μM), PDTC (100 μM), or both for indicated times. The endogenous JNK activity was determined by immunocomplex kinase assays

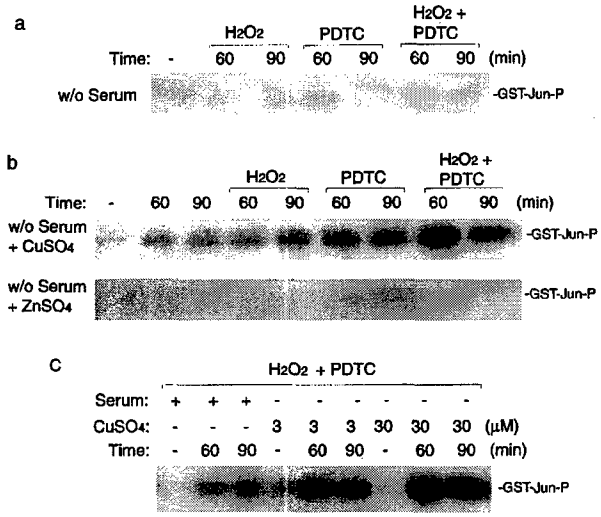

Figure 2 Copper but not zinc ions can replace serum, which is required for PDTC-induced JNK activation. HEK293 cells were cultured in serum-free medium (**a**) or serum-free medium containing $CuSO_4$ (30 μM) or $ZnSO_4$ (30 μM) (**b**) for 30 min, then were treated with H_2O_2 (100 μM), PDTC (100 μM) and H_2O_2 plus PDTC (100 μM each) for indicated times. (**c**) HEK293 cells were cultured in medium supplemented with 10% serum or serum-free medium containing $CuSO_4$ (3 or 30 μM) for 30 min, then were treated with H_2O_2 plus PDTC (100 μM each) for indicated times. Endogenous JNK activity was examined by immunocomplex kinase assays

PDTC. The addition of BCS suppressed JNK activation by PDTC or H_2O_2 plus PDTC in HEK293 cells, and suppressed JNK activation by H_2O_2 plus PDTC in Jurkat cells (Figure 3). BCS did not suppress UV-C-induced JNK activation in both HEK293 and Jurkat cells (data not shown), indicating that BCS did not suppress JNK activation nonspecifically. These data suggest that PDTC's metal ion-chelating ability plays an important role in JNK activation, and that the translocation of copper ions across the cytoplasmic membrane is essential for PDTC-induced JNK activation.

PDTC and H_2O_2 activate JNK in the absence of significant activation of SEK1/MKK4 and MKK7

H_2O_2 has been shown to activate apoptosis-activating kinase 1 (ASK1) (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998), a JNK-activating kinase. Therefore, oxidative stress may regulate JNK by activating the upstream kinases in the JNK pathway. To examine whether this is the case in PDTC plus H_2O_2-induced JNK activation, a GST-tagged SEK1, a JNK kinase, was transfected into HEK293 cells, and the transfected cells were subjected to PDTC plus H_2O_2 or UV-C treatments. The activities of SEK1 and JNK in treated cells were examined. Only a very weak SEK1 activation was detected in PDTC plus H_2O_2-treated cells, although an evident SEK1 activation was observed in UV-C-treated cells (Figure 4a). Both treatments induced significant JNK activation in HEK293 cells (Figure 4a). Similarly, PDTC plus H_2O_2 did not induce detectable MKK7 activation, while UV-C induced MKK7 activation (Figure 4b). This result suggests that activation of JNK upstream kinases is not the major cause of JNK activation by PDTC plus H_2O_2.

JNK inactivation rate is decreased in cells treated with PDTC plus H_2O_2

JNK activity is controlled by upstream kinases and by dual-specificity phosphatases that dephosphorylate and inactivate JNK. Activation of JNK upstream kinases, a decrease in JNK phosphatase activity, or the combination of both can lead to JNK activation. Since we did not observe significant activation of SEK1 and MKK7 (Figure 4), it is possible that JNK activation by PDTC and H_2O_2 was due to the decrease in JNK phosphatase activity and subsequently, the accumulation of JNK activity. To test this possibility, we examined the JNK inactivation rate by measuring the decrease of JNK activity post depletion of intracellular ATP, which prevented further activation of JNK by upstream kinases. HEK293 cells were exposed to PDTC plus H_2O_2 or UV-C for induction of JNK, then were treated with rotenone, an inhibitor of the respiratory chain and 2-deoxy-D-glucose, an inhibitor of glycolysis, for ATP depletion (Meriin *et al.*, 1999). Upon depletion of ATP, JNK activity was slowly decreased in cells treated with PDTC plus H_2O_2 (Figure 5). In contrast, JNK activity was rapidly decreased post depletion of ATP in cells treated with UV-C (Figure 5). These results suggest that the inactivation rate of JNK is much slower in cells treated with PDTC and H_2O_2 in comparison with that in UV-C-treated cells. Since JNK inactivation is mainly controlled by dual-specificity phosphatases, these results imply that these phosphatases may be down-regulated by PDTC and H_2O_2.

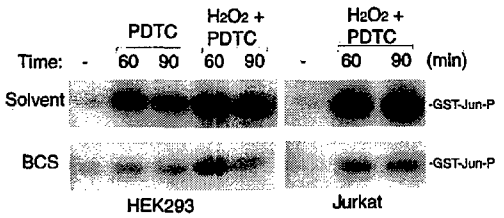

Figure 3 PDTC-induced JNK activation is inhibited by a nonpermeable cuprous ion chelator, BCS. HEK293 cells were incubated with media containing BCS (100 μM) for 30 min, then treated with PDTC (100 μM), H_2O_2 plus PDTC (100 μM each) for indicated times. Jurkat cells were incubated with media containing BCS (100 μM) for 30 min, then treated with H_2O_2 plus PDTC (100 μM each) as indicated. The endogenous JNK activity was determined by immunocomplex kinase assays

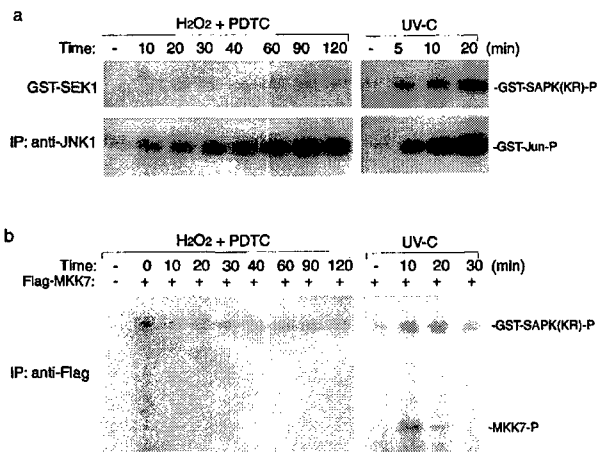

Figure 4 H_2O_2 and PDTC activated JNK in the absence of significant activation of JNK kinases. (**a**) HEK293 cells were transfected with GST-SEK1 and treated with H_2O_2 plus PDTC (100 μM each) or UV-C (200 J/m^2). Transfected GST-SEK1 was isolated by affinity purification using GSH-beads, and assayed for kinase activity using GST-SAPK(KR) as a substrate. Endogenous JNK activation was assayed by immunocomplex kinase assays. (**b**) HEK293 cells were transfected with Flag-MKK7 and treated with H_2O_2 plus PDTC (100 μM each) or UV-C (200 J/m^2). Transfected MKK7 was isolated by affinity purification using an anti-Flag antibody and protein G beads, and assayed for kinase activity using GST-SAPK(KR) as a substrate

PDTC and H_2O_2 induce the down-regulation of a JNK phosphatase M3/6

Many dual-specificity phosphatases are known to down-regulate JNK activity by dephosphorylating JNK at the critical Thr-Pro-Tyr motif (reviewed in Camps *et al.*, 1999). To test whether PDTC plus H_2O_2 activate JNK by down-regulating JNK phosphatases, we examined the effect of this treatment on JNK-specific phosphatase M3/6 (also named hVH-5; Camps *et al.*, 1999; Martell *et al.*, 1995; Muda *et al.*, 1996). PDTC plus H_2O_2 down-regulated the levels of transfected M3/6 in HEK293 cells (Figure 6a). The decrease in M3/6 levels coincided with the induction of

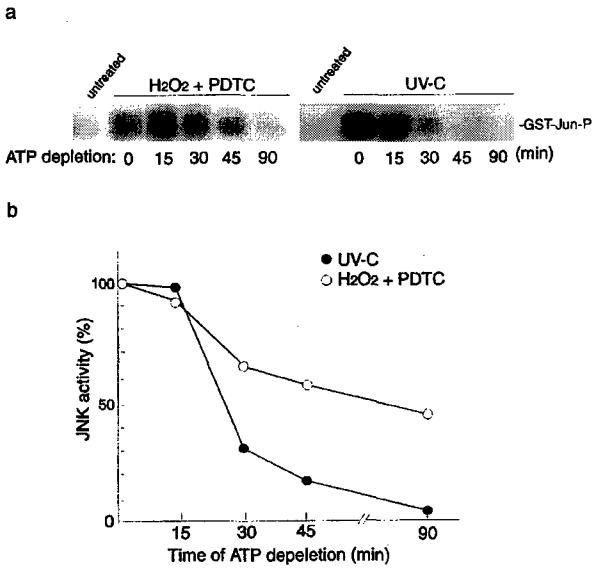

Figure 5 JNK inactivation rate is decreased in cells treated with PDTC plus H_2O_2. (**a**) HEK293 cells were exposed to UV-C (250 J/m^2), followed by a 30 min rest, or to PDTC plus H_2O_2 (100 μM each; 90 min), then were subjected to ATP depletion (10 μM rotenone and 25 mM 2-deoxy-D-glucose in PBS). Cells were collected at indicated time points after the ATP depletion. Endogenous JNK activity was examined by immunocomplex kinase assays. (**b**) Data in panel a were quantitated by a densitometer. JNK activity induced by UV-C or PDTC plus H_2O_2 at time 0 at ATP depletion was considered as 100%

JNK activity (Figure 6b). PDTC and H_2O_2 showed no effect on two other protein phosphatases, PP2A and PP4 (Figure 6a), indicating that the down-regulation in M3/6 was not a non-specific effect on any protein phosphatases. To examine if the down-regulation of M3/6 occurred in JNK activation induced by other stimuli, we tested the effect of UV-C on the expression of M3/6. We did not detect significant decreases in M3/6 levels after UV-C irradiation (Figure 6a).

The over-expression of M3/6 only slightly decreased the induction of JNK by PDTC plus H_2O_2 in the transfected HEK293 cells (Figure 6b). This observation is consistent with the result that M3/6 itself was down-regulated by PDTC plus H_2O_2 (Figure 6a). In contrast to what we observed in the PDTC and H_2O_2 treatment, over-expression of M3/6 significantly decreased the induction of JNK activity by UV-C irradiation (Figure 6b). These data indicate that M3/6 is targeted in the PDTC plus H_2O_2 treatment. However, our data do not rule out the possibility that other JNK phosphatases are also down-regulated by this treatment.

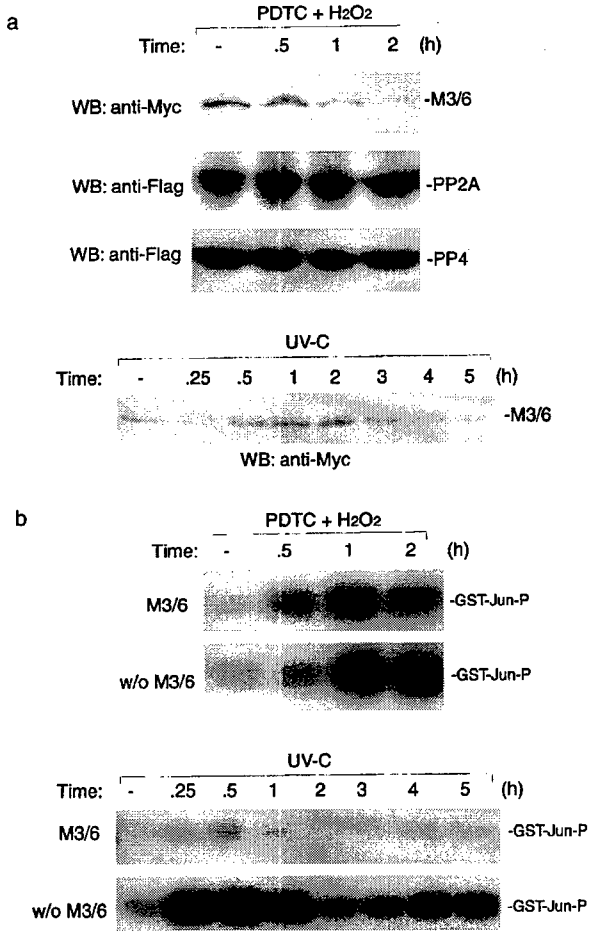

Figure 6 PDTC induced the down-regulation of a JNK phosphatase M3/6. (**a**) HEK293 cells were transfected with pBABEpuro-Myc-M3/6, Flagg-PP2A, or Flag-PP4. Transfected cells were treated with H_2O_2 plus PDTC (100 μM each) or UV-C (250 J/m^2). Cells were collected at various time points as indicated. Expressions of transfected genes were examined by Western blot using an anti-Myc or anti-Flag antibody. (**b**) HEK293 cells transfected with or without Myc-M3/6 were treated with H_2O_2 plus PDTC (100 μM each) or UV-C (250 J/m^2) as indicated. Endogenous JNK activation was examined by immunocomplex kinase assays

Discussion

PDTC, a presumed antioxidant, is widely used as an inhibitor for NF-κB activation (Beg *et al.*, 1993; Schreck *et al.*, 1992). In this study, we found that PDTC induced JNK activation by itself in certain cells, and activated JNK synergistically with exogenous H_2O_2. These observations and the fact that JNK is activated by oxidative stresses are contradictory to the assumption that PDTC is an antioxidant. Some other antioxidants, such as *N*-acetyl-L-cysteine and butylated hydroxyanisole, were also shown to activate JNK (del Arco *et al.*, 1996). However, prolonged exposure of cells to high doses of antioxidants has been linked with induction of endoplasmic reticular stress, which is a strong JNK activating signal, by causing protein misfolding (Urano *et al.*, 2000). Therefore, the activation of JNK by antioxidants needs to be carefully examined and interpreted.

Our results indicate that the induction of JNK by PDTC is actually due to its metal ion-chelating ability. PDTC is capable of binding to many metal ions (Gilman *et al.*, 1990), including copper, zinc, and iron, the three most abundant transition metal ions in serum (10–30 μM; Lentner, 1984). However, PDTC has only been shown to increase intracellular levels of copper and zinc, but not iron (Nobel *et al.*, 1995; Verhaegh *et al.*, 1997). Both copper and zinc are capable of binding to cellular proteins and may cause conformational and functional changes in proteins. In this study, we found that only copper ions, but not zinc ions, were capable of replacing serum in activation of JNK by PDTC and H_2O_2. Although the metal ions transported by PDTC may have multiple effects on cellular functions, a unique property of copper ions contributed to the JNK-activating ability. Since cuprous ions (Cu^+) can react with H_2O_2 to generate hydroxyl radicals (Halliwell and Gutteridge, 1990), we propose that PDTC may increase intracellular copper ions, and serve as a pro-oxidant that enhances production of hydroxyl radicals (Figure 7). However, it is still possible that copper ions may cause JNK activation through other mechanisms independent of ROS production.

In this study we found that the ability of H_2O_2 and PDTC to induce JNK activation varied in different cell types (Figure 1). This may be due to the variation in the production rate of H_2O_2 and the capacity to remove H_2O_2 by protective enzymes (e.g., catalase or peroxidase) among different cell types. In cells that have low H_2O_2 production and low capacity in removing H_2O_2, exogenous H_2O_2, but not PDTC, is likely to induce significant JNK activation. Conversely,

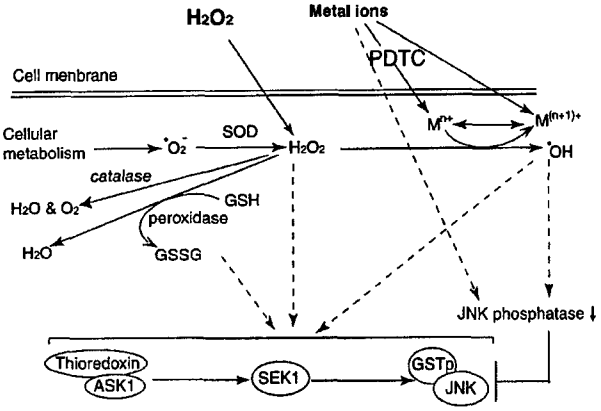

Figure 7 Model–Regulation of JNK by oxidative stress and metal ions. Intracellular redox status is determined by the rates of production and removal of ROS, including superoxide anions, H_2O_2, and hydroxyl radical. Transition metal ions, ferrous (Fe^{2+}) and cuprous (Cu^{+}) ions, facilitate the production of hydroxyl radical, the most reactive ROS, from H_2O_2. Exogenous H_2O_2 and metal ions can increase the production of hydroxyl radicals and cause damage to cells. PDTC promotes oxidative stress by increasing intracellular redox-reactive metal ions, such as cuprous ions. The JNK pathway can be activated by oxidative stress via the activation of upstream activator using the associated redox enzyme as sensors (Adler *et al*., 1999; Saitoh *et al*., 1998), and the down-regulation of JNK phosphatases. SOD, superoxide dismutase; GSH, glutathione; GSTp, glutathione S-transferase Pi

cells that have rapid H_2O_2 production and high enzymatic capacity in removing H_2O_2 are likely to be more resistant to exogenous H_2O_2. However, this type of cell may be more sensitive to PDTC, because copper ions imported by PDTC can generate hydroxyl radicals by reacting with endogenous H_2O_2 before H_2O_2 can be removed by cellular enzymes. Nevertheless, additional experiments are required to confirm this potential mechanism for cell type differences in responses to PDTC and H_2O_2.

Our results reveal that activation of JNK upstream kinases may not be the major cause of JNK activation by oxidative stress, since H_2O_2 plus PDTC induced very weak SEK1 activation and undetectable MKK7 activation. Like protein tyrosine phosphatases, dual-specificity phosphatases, which inactivate MAPKs including JNK, contain an essential catalytic cysteinyl residue (reviewed in Camps *et al*., 1999). However, different from tyrosine phosphatases, which have a deep catalytic cleft, dual-specificity phosphatases have shallow catalytic sites, which allow for the less stringent phospho-amino acid specificity (reviewed in Camps *et al*., 1999). This shallow catalytic surface may also expose the critical cysteinyl residue to oxidative damage. In this study, JNK activity induced by H_2O_2 plus PDTC had a much slower inactivation rate compared to that induced by UV-C (Figure 5), suggesting that H_2O_2 and PDTC may suppress JNK phosphatases. This result is consistent with a recent study reporting that protein-damaging stresses, including oxidative stress, inhibitor JNK dephosphorylation (Meriin *et al*., 1999). In addition, we found that M3/6, a dual-specificity phosphatase and a negative regulator of JNK (Muda *et al*., 1996), was down-regulated by this treatment (Figure 6). Down-regulation of M3/6 by H_2O_2 plus PDTC was a specific event, since protein phosphatases PP2A and PP4 were not affected by the same treatment, and UV-C irradiation did not cause the down-regulation of M3/6 (Figure 6a). It is possible that the down-regulation of JNK phosphatases is the major cause of JNK activation, since over-expression of M3/6 decreased the basal JNK activity and UV-induced JNK activation, but had little effect on JNK activation by H_2O_2 plus PDTC (Figure 6b). This result reveals that M3/6 is a potential target of PDTC and H_2O_2. It is possible that other dual-specificity phosphatases are also targeted by oxidative stress and by metal toxicity (Figure 7). Previously, we have proposed that lack of JNK phosphatases activity may be one of the causes of sustained JNK activation induced by apoptotic stimuli (Chen and Tan, 2000; Chen *et al*., 1996b). The data in this study and the fact that oxidative stress is observed in apoptosis induced by various stimuli (Fuchs *et al*., 1997; Jacobson, 1996) support the hypothesis that JNK phosphatases may be down-regulated during apoptosis.

Taken together, we observed a synergistic JNK activation by H_2O_2 plus PDTC, which may be mediated through the import of copper ions and subsequent down-regulation of JNK phosphatases. These data indicate that PDTC may have pro-oxidative effects in certain cellular contexts (e.g., high H_2O_2 production). Our data also reveal a need to re-evaluate the biological functions of PDTC, which has been widely used as an antioxidant and an NF-κB inhibitor (Beg *et al*., 1993; Schreck *et al*., 1992). Many observed phenomena could be due to the complex effects of the multiple functions of PDTC.

Materials and methods

Cell cultures and transfection

Human Jurkat T cells and embryonic kidney 293 (HEK293) cells were cultured as previously described (Chen *et al*., 1998b). LNCaP and Tsu-Pr1 human prostate cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For transient transfection in HEK293 cells, cells were plated at a density of 1.5×10^5 cells/35-mm well the day before transfection. Transfections were performed by a calcium phosphate precipitation method (Specialty Media, Phillipsburg, NJ, USA). The transfected cells were over 90% confluent 36–40 h after transfection, and were treated and harvested as indicated in individual experiments.

Antibodies and reagents

Rabbit anti-JNK1 antibodies (Ab101) were described previously (Chen *et al*., 1996a). Anti-Myc and anti-Flag antibodies were purchased from Boehringer Mannheim (Indianapolis, IN, USA) and Sigma (St. Louis, MO, USA), respectively. Protein A and protein G beads were purchased from Bio-Rad (Hercules, CA, USA) and Pharmacia (Piscat-

away, NJ, USA), respectively. H_2O_2, pyrrolidine dithiocabamate (PDTC), bathocuproinedisulfonic acid (BCS), $CuSO_4$, $ZnSO_4$, rotenone, and 2-deoxy-D-glucose were purchased from Sigma.

Plasmids

The pBABEpuro-Myc-M3/6 plasmid was a gift from A Smith, A Theodosiou and A Ashworth (The Institute of Cancer Research, London, UK) (Smith *et al.*, 1997), pMTSM-Myc-M3/6 plasmid was a gift from N Rodrigues and KE Davis (University of Oxford, Oxford, UK) (Theodosiou *et al.*, 1996), pBJ-Flag-protein phosphatase 2A (PP2A) plasmid was a gift from J Chen (University of Illinois, Urbana, IL, USA) (Chen *et al.*, 1998a), GST-SAPK(KR) plasmid was a gift from D Templeton (Case Western Reserve University, Cleveland, OH, USA), and the pEBG-SEK1 plasmid was a gift from L Zon (Harvard Medical School, Boston, MA, USA) (Sanchez *et al.*, 1994). The constructs for Flag-PP4 (PPX), GST-Jun(1–79), and Flag-MKK7 were described previously (Chen and Tan, 1998; Hu *et al.*, 1998; Yao *et al.*, 1997).

UV irradiation and depletion of cellular ATP

UV irradiation was performed using a UV Stratalinker 1800 (Stratagene). ATP depletion was performed as described with modifications (Meriin *et al.*, 1999). Cells were washed once with phosphate buffered saline (PBS) preheated to 37°C, and then incubated in PBS containing 10 μM rotenone and 25 mM 2-deoxy-D-glucose for indicated times.

Whole cell extracts preparation

Whole cell lysate was prepared by suspending 2×10^6 cells in 150 μl lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at 15 000 *g* for 10 min, and the supernatant was stored at −80°C.

In vitro *kinase assays*

JNK assays were performed as described previously (Chen *et al.*, 1998b). In brief, 50–100 μg of cellular proteins was incubated with specific antibody and protein A-agarose beads in lysis buffer at 4°C for 2 h for immunoprecipitation. The immunocomplexes were washed twice with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl [pH 7.6] and 0.1% Triton X-100), and twice with kinase buffer (20 mM 4-morpholinepropane-sulfonic acid [MOPS; pH 7.6], 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothrietol [DTT] and 1 mM Na_3VO_4). The washed complexes were then mixed in 30 μl of kinase buffer containing 50 μM of ATP, and 10 μCi of [γ-^{32}P]ATP. Four μg of GST-Jun(1–79) was added per reaction as a substrate. The reaction was performed at 30°C for 30 min, then terminated by adding SDS Sampling buffer. The reaction mixtures were boiled and analysed by SDS–PAGE and autoradiography. SEK1 assays were carried out similarly as JNK assays, except that the transfected GST-SEK1 was affinity purified by GSH-conjugated beads (Sigma), and the assays were performed using the recombinant GST-SAPK(KR) protein (4 μg per reaction) as a substrate.

Western blot analysis

For Western blot analysis, the samples were prepared as described above. The samples (50–100 μg) were resolved by SDS–PAGE, and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with a primary antibody (anti-Myc, 1 : 1000; anti-Flag, 1 : 1000), washed twice, blotted with a secondary antibody conjugated with horseradish peroxidase (1 : 1000 dilution), and washed extensively. The membrane was then developed in the ECL reagent (Amersham, Arlington Heights, IL, USA) or the Super Signal Pico reagent (Pierce, Rockford, IL, USA), and exposed to an X-ray film.

Abbreviations

BCS, bathocuproinedisulfonic acid; HEK293 cells, human embryonic kidney 293 cells; GSH, glutathione; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; PDTC, pyrrolidine dithiocarbamate; PP2A, protein phosphatase 2A; PP4, protein phosphatase 4; redox, reduction-oxidation; ROS, reactive oxygen species; SEK1, SAPK/ERK kinase 1; UV-C; ultraviolet C.

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