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The Mitogen-activated Protein Kinase Phosphatases PAC1, MKP-1, and MKP-2 Have Unique Substrate Specificities and Reduced Activity *in Vivo* toward the ERK2 *sevenmaker* Mutation*

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Mitogen-activated protein (MAP) kinases can be grouped into three structural families, ERK, JNK, and p38, which are thought to carry out unique functions within cells. We demonstrate that ERK, JNK, and p38 are activated by distinct combinations of stimuli in T cells that simulate full or partial activation through the T cell receptor. These kinases are regulated by reversible phosphorylation on Tyr and Thr, and the dual specific phosphatases PAC1 and MKP-1 previously have been implicated in the *in vivo* inactivation of ERK or of ERK and JNK, respectively. Here we characterize a new MAP kinase phosphatase, MKP-2, that is induced in human peripheral blood T cells with phorbol 12-myristate 13-acetate and is expressed in a variety of nonhematopoietic tissues as well. We show that the *in vivo* substrate specificities of individual phosphatases are unique. PAC1, MKP-2, and MKP-1 recognize ERK and p38, ERK and JNK, and ERK, p38, and JNK, respectively. Thus, individual MAP kinase phosphatases can differentially regulate the potential for cross-talk between the various MAP kinase pathways. A hyperactive allele of ERK2 (D319N), analogous to the *Drosophila sevenmaker* gain-of-function mutation, has significantly reduced sensitivity to all three MAP kinase phosphatases *in vivo*.

Mitogen-activated protein (MAP)[1] kinases are activated by dual phosphorylation on Tyr and Thr, and they are important in mediating signal transduction from the cell surface to the nucleus (1). Several MAP kinase signal transduction pathways have been molecularly characterized (for reviews, see Refs. 2–6). In yeast, at least four genetically distinct, functionally independent, MAP kinase-mediated signaling pathways have been defined (3, 4).

In mammals, three structural families of MAP kinases have been identified that have distinct substrate specificities (4–6). The members of one family, ERK1 and ERK2, which are most closely related to yeast FUS3 and KSS1, are activated by growth and differentiation factors and by phorbol esters (7). The JNK family (also known as SAPK) and p38 MAP kinase (which is the closest structural homolog of the yeast HOG1) are activated either in common or parallel pathways by proinflammatory cytokines and environmental stress and to a lesser extent by growth factors such as EGF (8). Some stimuli, such as T cell receptor ligation and short wavelength ultraviolet light treatment, activate both ERK and JNK pathways (9, 10).

ERK, JNK, and p38 are regulated by reversible dual phosphorylation within the motifs TEY, TPY, and TGY, respectively (5). ERK2 has been shown to be dephosphorylated *in vitro* and *in vivo* on Thr and Tyr by the nuclear localized phosphatases PAC1 (11) and MKP-1 (12). In addition, JNK appears to be a target for MKP-1 (10). PAC1 and MKP-1 share highly homologous C-terminal catalytic domains but display relatively unique domains at their N termini, which may be involved in substrate recognition or possibly may be important in some aspects of post-translational regulation such as regulated proteolysis (13). PAC1 is expressed predominantly in hematopoietic cells, absent in quiescent cells, and transcriptionally induced within minutes following antigen receptor ligation or treatment with phorbol ester (13). MKP-1 is expressed in a wide variety of tissues and induced by growth factors and genotoxic and environmental stresses (10, 14, 15).

Recently, two additional MAP kinase phosphatases, hVH3 (16) (also known as B23 (17)) and MKP-2 (18) (also known as hVH2 (19)), have been isolated by virtue of their structural homology to PAC1 and MKP-1. MKP-2 and hVH3 display highly conserved catalytic and more distantly related N-terminal domains with PAC1 and MKP-1. MKP-2 and hVH3 have been shown to dephosphorylate ERK2 (16–19). *PAC1*, *MKP-1*, *hVH3*, and *MKP-2* are early response genes that have unique but overlapping tissue distributions (13, 15, 19). In order to begin dissecting the biological roles played by the related phosphatases PAC1, MKP-1, and MKP-2, we have determined in fine detail their *in vivo* substrate specificity for the three families of MAP kinases.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate and phytohemagglutinin were from Sigma; EGF and tumor necrosis factor α were from Peprotech; rabbit anti-ERK2 antibodies were from Upstate Biotechnology, Inc., and anti-CD3 monoclonal antibody was from Boehringer Mannheim. pMT2T-HA-p54β (JNK2) was provided by J. R. Woodgett (Ontario Cancer Institute); and pCEV-HA-HOG (p38), pcDNA3-HA-ERK2, GST-c-Jun-(1–79) and GST-ATF2-(1–96) plasmids were provided by J. S. Gutkind (NIDR, National Institutes of Health). Plasmids expressing PAC1, MKP-1, and MKP-2 were constructed by inserting their cDNA fragments into pMT2T. The polymerase chain reaction fragment of human ERK2 was cloned into the *Bam*HI site of pCGN-Hygro, and its *sevenmaker* gain-of-function mutation was made by replacing the Asp residue corresponding to codon 319 with an Asn residue.

Cell Culture and Transfection—COS-7, NIH3T3, and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and transfected by using lipofectamine (Life Technol-

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[1] The abbreviations used are: MAP, mitogen-activated protein; ATF2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MKP, MAP kinase phosphatase; HA, hemagglutinin; GST, glutathione *S*-transferase; EGF, epidermal growth factor; TCR, T cell receptor.

ogies, Inc.) according to the manufacturer's protocol. Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and transfected by using electroporation. Forty-eight h after transfection, cells were serum starved for 30 min and then stimulated as indicated.

MAP Kinases Assay—The stimulated cell lysates were prepared as described (11). The HA-tagged kinase was immunoprecipitated with monoclonal antibody 12CA5 directed against the HA epitope (20). The immunocomplex kinase assay was performed as described (21) using individual kinase substrates as indicated. After SDS-polyacrylamide gel electrophoresis, phosphorylated proteins were visualized by autoradiography.

Metabolic Labeling, Immunoprecipitation, and Western Blotting—Human peripheral blood T cells were purified and metabolically labeled as described previously (13). Cell lysates were prepared as above. The use of monoclonal and polyclonal antibodies against PAC1 has been described (13). MKP-2 was immunoprecipitated for 4 h with affinity-purified rabbit antibodies (13) directed against MKP-2 peptides (encompassing amino acids 12–27 and 128–142). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by either autoradiography or Western blotting.

In order to determine expression of HA-ERK2 and mutant HA-ERK2(D319N), cell lysates were immunoprecipitated for 2 h with the specific antibody 12CA5. The immunoprecipitates were analyzed by Western blots with enhanced chemiluminescence detection (Amersham Corp.) using rabbit anti-ERK2 antibodies.

RESULTS

Previous work by us (22) and others (23) has shown that there is an extended family of genes with homology to the MAP kinase phosphatases *PAC1* and *MKP-1*. Using low stringency hybridization of a *PAC1* coding region probe, we isolated and characterized a MAP kinase phosphatase-like gene from a human, mitogen-activated T cell cDNA library (24). Because this gene was most closely related to *MKP-1* and the encoded protein had *in vivo* MAP kinase phosphatase activity, we named it MAP kinase phosphatase 2 (*MKP-2*). During preparation of this paper, the sequences of *hVH2* and rat *mkp-2* were published (18, 19). *hVH2* is identical in the encoded amino acid sequence to our human MKP-2 and varies in 20 out of a total of 2368 nucleotide residues (see GenBank™ accession numbers U21108 and U48807). Rat MKP-2 is 89% homologous to human MKP-2 and unrelated between amino acids 97 and 131 due to a frameshift in the rat gene relative to human *MKP-2* and other MAP kinase phosphatases (16, 17).

Northern blot analyses demonstrated a low but detectable level of *MKP-2* mRNA in resting human peripheral blood T cells that was induced approximately 5-fold following stimulation by phorbol 12-myristate 13-acetate for 1 or 3 h (not shown). Two mRNA species of 6.0 and 2.3 kilobases were detected using an *MKP-2*-specific 3′-untranslated region probe (see Fig. 2). Because the probe detects a single gene in Southern blot analyses (not shown), the two mRNA species appear to result from alternative splicing or differential use of polyadenylation sites.

MKP-2 encodes a protein of 394 amino acids with a predicted molecular mass of 42.9 kDa. In order to verify the deduced sequence of MKP-2, we prepared anti-peptide antibodies, which were used to immunoprecipitate extracts derived from either resting or phorbol 12-myristate 13-acetate-activated, metabolically labeled human peripheral blood T cells (Fig. 1). The anti-peptide antibodies precipitated an easily detectable 42 kDa protein from activated but not resting peripheral blood T cells. The endogenous MKP-2 protein from peripheral blood T cells migrated identically to the protein specifically precipitated from extracts of COS-7 cells transfected with an MKP-2 expression vector (not shown).

In order to determine the tissue specificity of *MKP-2* expression, we probed Northern blots of multiple human tissue poly(A)$^+$ RNAs. As shown in Fig. 2, all of the 13 tissues examined expressed some level of *MKP-2* mRNA. Both 6.0- and 2.3-kilobase MKP-2 mRNA species were expressed in all tissues, but the relative concentration of the 2.3-kilobase band was higher in testis than other tissues. Prostate, testis, pancreas, adrenal cortex, and stomach displayed the highest relative levels. Common features of these tissues include a predom-

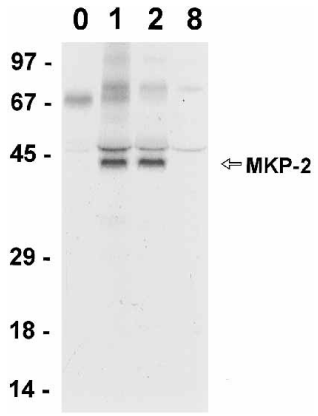

FIG. 1. **MKP-2 induction in human T cells.** Human peripheral blood T cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) and phytohemagglutinin (1 μg/ml) and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 h prior to harvesting. Cellular extracts were immunoprecipitated with a mixture of antibodies directed against MKP-2 peptides (encompassing amino acids 12–27 and 128–142). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Stimulation times (in h) are shown above each *lane*. Molecular mass makers are shown in the *left margin* in kDa.

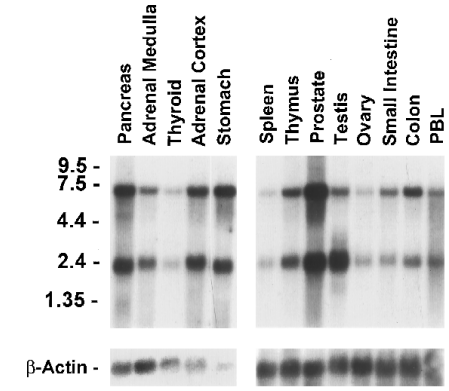

FIG. 2. ***MKP-2* is widely expressed in human tissues.** Human multiple tissue poly(A)$^+$ RNA blots (Clontech) were probed with a fragment corresponding to the 3′-untranslated region of *MKP-2* (nucleotides 1203–2241, GenBank™ accession number U48807) by using the QuickHyb method (Stratagene). RNA markers (in kilobases) are shown in the *left margin*. *PBL*, peripheral blood leukocytes.

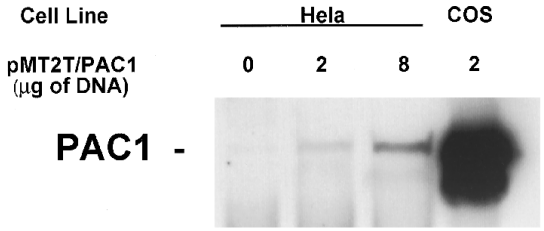

FIG. 3. **Protein expression is proportional to the amount of PAC1 cDNA transfected in HeLa cells.** HeLa or COS cells were grown and transfected with different amounts of PAC1 cDNA plasmid as indicated at the *top* of the figure. Forty-eight h after transfection the cells were harvested and extracts were made as described under "Experimental Procedures." Extracts of HeLa cells (5.6 mg of proteins) and COS cells (1.2 mg of proteins) were immunoprecipitated with a mixture of monoclonal antibodies (P9D10, P10C5, and P12B11) directed against PAC1. The immunoprecipitates were analyzed by Western blots with enhanced chemiluminescence detection (Amersham Corp.) using affinity-purified rabbit anti-PAC1 antibodies.

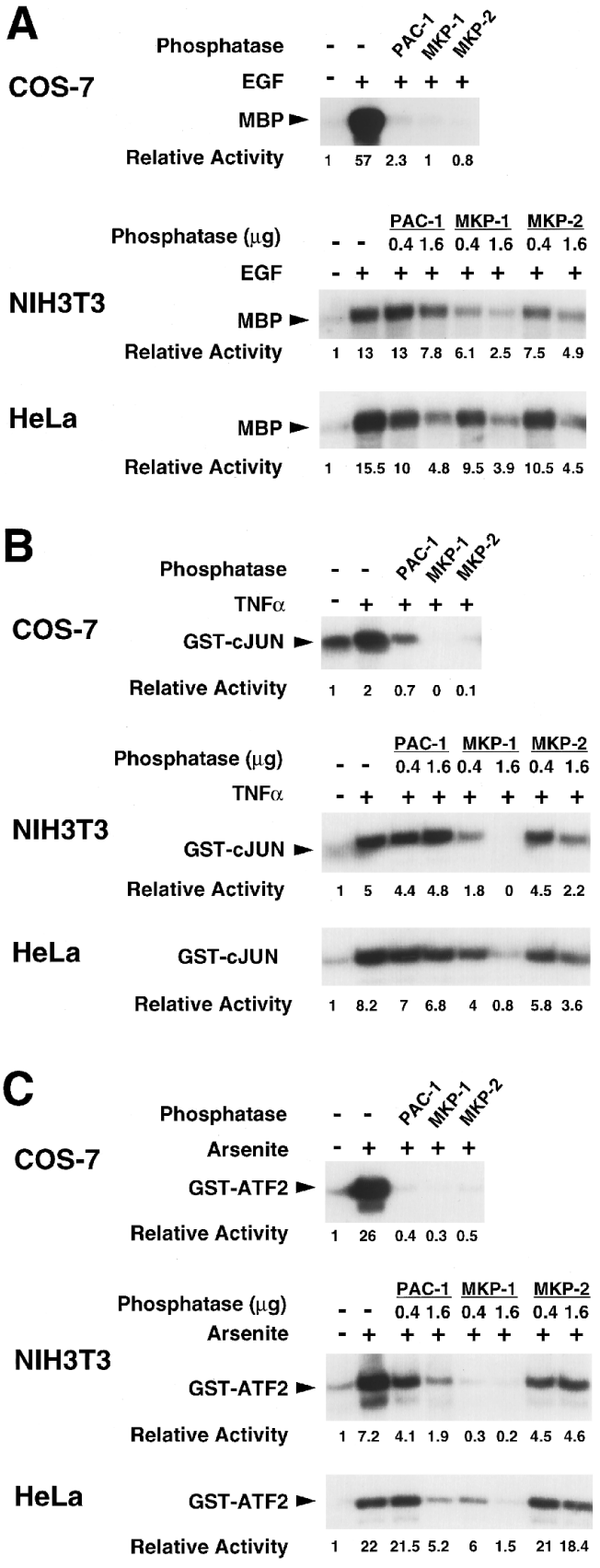

FIG. 4. **PAC1, MKP-1, and MKP-2 have distinct substrate specificities.** COS-7, NIH3T3, or HeLa cells were cotransfected with HA-ERK2 (*A*), HA-JNK2 (*B*), or HA-p38 (*C*) expression plasmids (0.4 μg) and PAC1, MKP-1, or MKP-2 expression plasmid (0.4 μg or 1.6 μg as indicated). After 48 h cells cotransfected with HA-ERK2 DNA were stimulated with EGF (25 ng/ml) for 5 min. Immunopurified HA-ERK2 was assayed for kinase activity by using myelin basic protein as a substrate. Cells cotransfected with HA-JNK2 DNA were stimulated with tumor necrosis factor α (*TNFα*) (50 ng/ml) for 15 min, and the activity of the immunopurified HA-JNK2 was measured by phosphorylation of GST-c-Jun-(1–79). Cells cotransfected with HA-p38 plasmid were stimulated with sodium arsenite (0.5 mM) for 30 min. Activity of the immunopurified HA-p38 was measured by using GST-ATF2-(1–96) as a substrate. The relative kinase activity was quantitated based on the activity present in unstimulated cells cotransfected with the MAP kinase plasmid and empty pMT2T.

inantly epithelial cell population and hormone responsiveness. Hormones such as cholecystokinin/gastrin (25, 26) that act upon pancreatic acini, gastrointestinal tract, and central nervous system, and angiotensin II (27) that acts upon adrenocortical cells have been shown to activate the ERK pathway upon binding to their receptors. It will be interesting to determine whether MKP-2 RNA induction is responsive to appropriate hormones in these tissues. Unlike MKP-1 and hVH3 (B23) (16, 17), *MKP-2* is not expressed following mitogenic or stress stimulation of human fibroblasts (not shown).

PAC1 and MKP-2 are expressed in T cells with overlapping kinetics following stimulation with phorbol 12-myristate 13-acetate. What might the distinct roles of these proteins be? We considered fine substrate recognition to be a likely possibility. Therefore, we assayed the relative ability of MKP-2, PAC1, and MKP-1 to dephosphorylate ERK2 and the other related MAP kinases, JNK2 and p38. COS-7, HeLa, and NIH3T3 cells were cotransfected with DNA encoding an epitope-tagged kinase and various concentrations of DNA encoding one of the MAP kinase phosphatases. As shown for PAC1 expression in HeLa cells, the transfection conditions used here resulted in a linear relationship between the amount of DNA transfected and the consequent levels of protein expression (Fig. 3). Following stimulation of the cells, the level of kinase activity achieved in the constitutive presence of the various MAP kinase phosphatases was determined.

Fig. 4*A* shows the activity of immunoprecipitated ERK2 following EGF stimulation as determined by myelin basic protein phosphorylation. COS-7 cells, which greatly overexpress the transfected proteins, demonstrated virtually complete inactivation of ERK2. However, the COS-7 system is not a good reflection of physiological MAP kinase phosphatase concentrations. Western blot quantitation of immunoprecipitated cellular extracts from cells transfected with PAC1 demonstrated that COS-7 cells expressed in excess of 1000 times more PAC1 than equivalently transfected HeLa cells (Fig. 3). As shown for HeLa cells in Fig. 4*A*, PAC1, MKP-1, and MKP-2 demonstrated similar degrees of ERK2 phosphatase activity that were in a titratable range. PAC1 had somewhat less activity in NIH3T3 cells relative to MKP-1 and MKP-2, which is probably a result of the instability of PAC1 in serum-starved NIH3T3 cells (not shown).

Following tumor necrosis factor α treatment of cells, the kinase activity of immunoprecipitated JNK2 was determined by its ability to phosphorylate the N-terminal fragment (1–79) of c-Jun (Fig. 4*B*). MKP-1 had robust, titratable activity against JNK2. MKP-2 had detectable activity against JNK2, although full inactivation of JNK2 was not observed even at the higher phosphatase concentration. Finally, PAC1 appeared to be inactive against JNK2 in NIH3T3 and HeLa cells and had minimal activity in COS-7 cells.

A third pattern of substrate specificity is seen in Fig. 4*C*, which shows the activity of immunoprecipitated p38 following arsenite treatment as assayed by the phosphorylation of the N-terminal fragment (1–96) of ATF2. All three phosphatases demonstrated activity against p38 in the COS-7 system. In NIH3T3 and HeLa cells MKP-1 was highly active against p38,

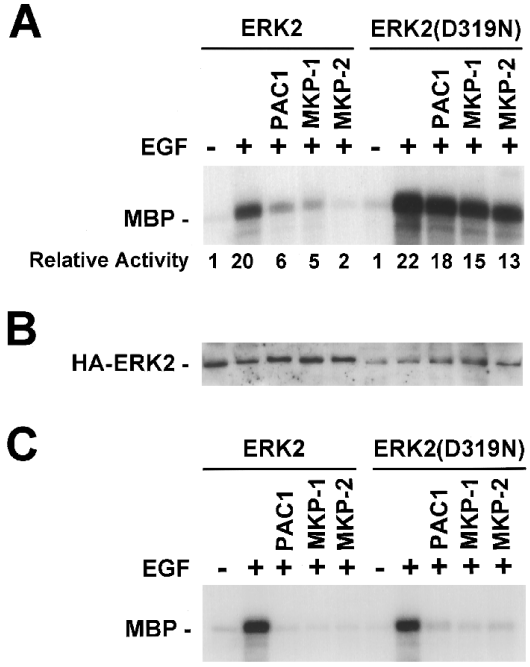

FIG. 5. **Mutant ERK2(D319N) is less sensitive to inactivation by MAP kinase phosphatases *in vivo*.** HA-ERK2 or mutant HA-ERK2(D319N) expression plasmid (1.25 μg) was cotransfected into cells with PAC1, MKP-1, or MKP-2 expression plasmid (10 μg). After 48 h transfected cells were stimulated with EGF (25 ng/ml) for 5 min. *A*, ERK2 activity was measured in transfected HeLa cells. The kinase activity was measured by immunocomplex kinase assays with myelin basic protein (*MBP*) (5 μg) as a substrate. The relative kinase activity was quantitated based on the activity present in unstimulated cells. *B*, Western blotting of ERK2 in transfected HeLa cells is shown. The 12CA5 immunoprecipitates were subjected to Western blotting using rabbit anti-ERK2 antibodies. *C*, ERK2 activity was measured in transfected COS-7 cells as described for *A*.

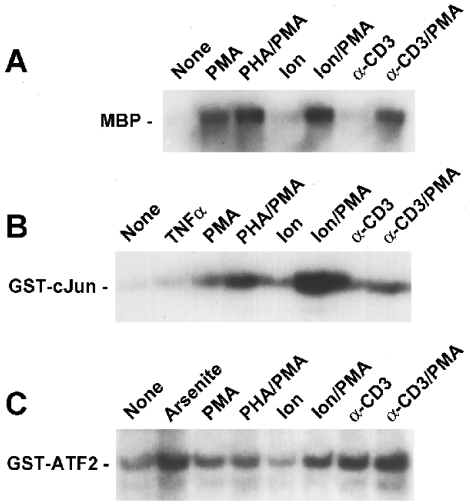

FIG. 6. **ERK2, JNK2, and p38 kinase activity is induced by immune stimulants in Jurkat cells.** Jurkat cells were transfected with HA-ERK2 (*A*), HA-JNK2 (*B*), or HA-p38 (*C*) plasmids by using electroporation. After 48 h cells were stimulated as indicated with tumor necrosis factor α (*TNF*α, 50 ng/ml), sodium arsenite (0.5 mM), phorbol 12-myristate 13-acetate (*PMA*, 50 ng/ml), phytohemagglutinin (*PHA*, 1 μg/ml), ionomycin (*Ion*, 2 μg/ml), anti-CD3 (10 μg/ml), or combinations of the above as indicated. Activity of the HA-tagged kinases was measured using immunocomplex kinase assays with substrates as indicated. *MBP*, myelin basic protein.

while PAC1 showed moderate levels of titratable activity. MKP-2 had virtually no activity against p38 in NIH3T3 and HeLa cells, indicating that activity toward p38 is observed only at high, nonphysiological concentrations of MKP-2. In considering all three substrates, each individual phosphatase showed a unique pattern of substrate specificity.

We examined the activity of PAC1, MKP-1, and MKP-2 toward a hyperactive mutant of mammalian ERK2 (D319N ERK2) that is analogous to the *Drosophila sevenmaker* gain-of-function mutation (28) and contains a substitution of Asn for Asp at position 319. D319N ERK2 does not have constitutive kinase activity *in vitro* but instead appears to be more highly responsive to activation (29), possibly as a result of decreased sensitivity to phosphatases. In order to determine the sensitivity of D319N ERK2 to MAP kinase phosphatases *in vivo*, we utilized the cotransfection system described above. As shown in Fig. 5*A*, D319N ERK2 was resistant to inactivation by levels of PAC1, MKP-1, and MKP-2 that inactivated wild type ERK2 to near background levels. This effect was not due to increased expression of D319N ERK2 relative to that of wild type ERK2 as demonstrated by Western blotting of epitope-tagged ERK2 and D319N ERK2 (Fig. 5*B*). In addition, EGF-stimulated increases in D319N ERK2 activity were completely eliminated in COS-7 cells that had been cotransfected with a MAP kinase phosphatase (Fig. 5*C*), demonstrating the sensitivity of the *sevenmaker* mutation at sufficiently high MAP kinase phosphatase concentrations.

In order to address the potential physiological function of the MAP kinase phosphatases induced in T cells, we determined whether there is a correlation between the stimuli that activate the different kinases and the stimuli that induce the phosphatases. PAC1 and MKP-2 are induced within the 1st h after activating T cells with mitogens (Fig. 1 and Ref. 13) or T cell receptor (TCR) ligation (30). We have been unable to detect MKP-1 protein in activated T cells. Stress stimuli (including short wavelength ultraviolet light, arsenite, and hyperosmolarity) that activate the JNK and p38 kinases did not induce PAC1 or MKP-2 in T cells (not shown). Previous results have shown that ERK2 and JNK1 are activated following T cell activation (9). Therefore, we investigated the range of stimuli that induce p38, ERK, and JNK activation in T cells.

We assayed the catalytic activation of epitope-tagged, transfected ERK2, JNK2, and p38 in Jurkat cells following various types of stimulation. α-CD3 binds the TCR and is partially stimulatory, while the combination of phorbol 12-myristate 13-acetate and ionomycin mimics co-stimulation through the TCR and accessory molecules. As shown in Fig. 6*A*, ERK2 was maximally activated with phorbol 12-myristate 13-acetate alone, consistent with the previous results (31), and the conditions of α-CD3 stimulation used here were nonactivating for ERK2. A different pattern of activation was seen with JNK2 (Fig. 6*B*). JNK2 was weakly stimulated by phorbol 12-myristate 13-acetate alone, stimulated to a greater extent with phorbol 12-myristate 13-acetate in combination with α-CD3 or phytohemagglutinin, and maximally stimulated by co-stimulation with phorbol 12-myristate 13-acetate and ionomycin. p38 was activated by TCR ligation in a manner distinct from ERK and JNK (Fig. 6*C*). p38 was not activated by phorbol 12-myristate 13-acetate alone, was stimulated to near maximal activity with α-CD3, and was stimulated maximally by the combination of phorbol 12-myristate 13-acetate and ionomycin or α-CD3.

DISCUSSION

MAP kinase phosphatases form a family characterized by highly structurally related catalytic domains of approximately 15 kDa (32). The known MAP kinase phosphatases are early response genes and have unique but highly overlapping tissue distributions (13, 15, 19). We show here that PAC1, MKP-1, and MKP-2 have distinct patterns of substrate specificity. MKP-1-inactivated ERK2, JNK2, and p38 equally. PAC1 did

not inactivate JNK2, nor did MKP-2 inactivate p38. It is not possible to make a statement about the relative specific activity of the various phosphatases. However, it is clear that individual phosphatases demonstrated unique patterns of MAP kinase recognition within a single cell system. In addition, the results were consistent between NIH3T3 and HeLa cells. MAP kinase phosphatases appear to exist in cells at low steady state concentrations (11, 15) that were approximated by the transfection conditions used here for NIH3T3 and HeLa cells. At high concentrations of PAC1, MKP-1, and MKP-2 such as exists in transfected COS-7 cells, much of the substrate specificity is lost (see Figs. 3–5).

We exploited the cotransfection system described above to investigate the specificity of the various phosphatases toward a hyperactive allele of ERK2 (D319N ERK2). The substitution of Asn for Asp at position 319 of mammalian ERK2 is equivalent to the *Drosophila sevenmaker* gain-of-function mutation. D319N ERK2 demonstrated almost no sensitivity toward dephosphorylation by PAC1, MKP-1, or MKP-2 under conditions that inactivated wild type ERK2 to background levels. D319 is located on the exterior face of ERK2 on the opposite side of the molecule from the phosphorylation lip and substrate binding site (33). The substitution of Asn for Asp at position 319 has been proposed to affect the structure or orientation of the N and C domains (33). Interestingly, the D319N mutation does not appear to greatly affect the catalytic activity of ERK2 or its recognition by MAP kinase kinase (29). D319N ERK2 can be dephosphorylated by the high concentrations of phosphatases expressed in COS-7 cells *in vivo* (Fig. 5*C*) or by purified MKP-1 *in vitro* (29), suggesting that there is not an absolute structural impediment to dephosphorylation. Therefore, most likely the affinity of the MAP kinase phosphatases for D319N ERK2 is sufficiently decreased to have a substantial effect *in vivo*.

T cell activation results in ERK, JNK, and p38 activation, which is followed by induction of PAC1 and MKP-2 expression. The three kinases are responsive to distinct combinations of stimuli, suggesting the potential for differential regulation of the kinase pathways in response to external signals in T cells. Thus, various conditions of immune stimulation such as activation or tolerance induction may lead to differential activation of ERK, JNK, and p38.

TCR-mediated stimulation activates multiple MAP kinase pathways, as appears to be the case for short wavelength ultraviolet light treatment (9, 34) and probably a variety of other stimuli as well. It seems likely that targets of the various MAP kinase pathways will cooperate to affect gene expression or other physiological responses to extracellular stimuli. Differential induction of the MAP kinase phosphatases in a cell type or situation-specific manner appears to contribute an additional level of regulation upon the relative activities of the various MAP kinase pathways.

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