Regulation of Dual-specificity Phosphatases M3/6 and hVH5 by Phorbol Esters

**ANALYSIS OF A DELTA-LIKE DOMAIN**

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Treatment of leukemic cells with phorbol 12-myristate 13-acetate (PMA) induces a short-lived phosphorylation and activation of stress-activated protein kinase (SAPK) and cellular differentiation. To investigate whether the rapid deactivation of SAPK results from dephosphorylation by dual-specificity phosphatases (DSPs), we studied regulation of the DSP hVH5 and its murine orthologue M3/6 in K562 human leukemia cells. PMA treatment rapidly induced hVH5 transcripts in these cells, and induced expression of M3/6 completely inhibited PMA-stimulated phosphorylation of SAPK, suggesting a feedback loop to control SAPK activity. Using both stable cell lines and transient transfection we demonstrate that activation of SAPK rapidly stimulated phosphorylation of M3/6. This phosphorylation did not regulate the half-life of total cellular M3/6; hVH5 and M3/6 shares with all sequenced mammalian DSPs an amino acid motif, XILPXLXL, located approximately 80 amino acids from the active site. The hVH5-M3/6 sequence, RILPHLYL, shares significant homology with the SAPK binding site of the c-Jun protein, called the delta domain. This motif was found to be important for DSP function, because deletion of RILPHLYL inhibits SAPK-mediated phosphorylation of M3/6, and deletion of this sequence or mutation of the LYL portion blocks the ability of this phosphatase to dephosphorylate SAPK.

Signals transduced by mitogen-activated protein kinase (MAPK) cascades exert profound effects on mitosis, cellular differentiation, and apoptosis. The addition of phorbol esters, e.g. phorbol 12-myristate,13-acetate (PMA) to hematopoietic cells activates these protein kinases and induces both cellular differentiation and apoptosis. In particular, PMA treatment causes the megakaryocytic differentiation of K562 cells (1). Thus far, five MAPK cascade pathways have been identified in eukaryotic cells, of which three have been relatively well characterized: the extracellular signal-regulated kinase (ERK) pathway; the stress-activated protein kinase (SAPK) or c-Jun amino-terminal kinase (JNK) pathway; and the p38 pathway (reviewed in Refs. 2 and 3). The complexity of cellular responses to activation of these pathways, many of which are cell type-specific, has generated intense interest in how cells regulate the activity of these individual protein kinase cascades.

Regulation of the SAPK pathway is of particular interest, because SAPK in some contexts appears to play an obligatory role in apoptosis and embryonic development. Dual knockouts in mice of JNK 1 and JNK 2 cause embryonic lethality due to severe dysregulation of apoptosis during brain development (4), the defect being either pro- or anti-apoptotic depending on the brain region. *Drosophila* mutants which lack the homologue of either SAPK (DJNK) or its activator kinase (5, 6) die at an early stage with a failure of the dorsal epidermis to close over the amnioserosa. Interestingly, DJNK increases expression of Puckered, a dual-specificity phosphatase, which appears to participate in a feedback loop to control DJNK activity (7).

Although some tyrosine phosphatases are known to act specifically on members of the MAPK cascades (e.g. Ref. 8), the gene family of dual-specificity phosphatases (DSPs) has emerged as a prime candidate for *in vivo* negative regulation of the MAPKs (reviewed in Refs. 9 and 10). The prototypical DSP, VHI, was originally identified as an open reading frame in vaccinia virus (11); the bacterially expressed protein was shown to be capable of dephosphorylating protein substrates containing both phosphotyrosine and phosphoserine residues. Subsequently, the crystal structure was determined, which showed strong secondary structural links with mammalian DSPs and the phosphatase and tensin homolog deleted on chromosome ten (PTEN) (12). At this point, nine human DSPs are known. All DSPs share the phosphotyrosine catalytic site consensus sequence (IV)HXAGXR(S/T)G, strong homologies in a region of approximately 140 residues encompassing the catalytic site, and short regions of homology with the cell cycle regulatory protein cdc25 (CH2 domain (13)). A number of DSP genes undergo transcriptional up-regulation in response to growth factors and/or cellular stress (summarized in Ref. 9); in several instances the ERK or SAPK cascades have been implicated in this response (MKP-1: Refs. 14, 15; PAC-1: Ref. 16; M3/6: this paper). With a few exceptions, the substrate specificities of the DSPs appear to be broad, acting approximately equally on ERK, SAPK, and p38. The exceptions are MKP-1, which *in vivo* in U937 cells preferentially dephosphorylates p38 and SAPK (14); MKP-3 (Pyst1), which has a preference for ERK and is activated by binding to ERK2 (17, 18); MKP-5, which preferentially dephosphorylates SAPK and p38 (19, 20); and hVH5, which also has a strong preference for SAPK and p38 (22).

Previously, we demonstrated that MKP-1 is induced by PMA treatment of U937 cells, suggesting a feedback regulation of
SAPK/JNK activity. Using MAPK inhibitors, we demonstrated that PMA-induced regulation of MKP-1 is most likely mediated by ERK2 activation (14). Because MKP-1 is a member of the DSP family, we have investigated whether other members of this family play a role in regulating the SAPK/JNK pathway in hematopoietic cells. Using K562 leukemia cells, we demonstrate that PMA-induced stimulation of the SAPK pathway causes marked increases in the steady-state levels of the hVH5 mRNA. We utilized M3/6, the murine orthologue of hVH5, for further analysis of specificity and regulation. hVH5 and M3/6 are 90% identical at the amino acid level (21), excluding a discrete 45-amino acid sequence present in M3/6 that is composed mainly (44/45 residues) of glycine and serine, as a result of a 138-bp complex trinucleotide repeat distal to the catalytic domain, which is absent from the hVH5 cDNA sequence. Induced expression of transfected M3/6 protein in K562 cells completely inhibits the PMA-induced activation of SAPK and partially blocks the activation of p38 protein kinase but has no effect on ERK kinase, demonstrating that this family of enzymes could have an essential role in SAPK/JNK activity regulation. In addition, PMA activates SAPK to phosphorylate the M3/6 protein. Unlike MKP-1 (28), this phosphorylation does not appear to change the half-life of M3/6 protein. M3/6 contains a region of similarity to the c-Jun SAPK interaction domain, the delta-like domain. Deletion or mutation of this domain in M3/6 prevents the SAPK-mediated phosphorylation of this protein and/or blocks the ability of M3/6 to dephosphorylate SAPK. Examination of multiple DSP sequences demonstrates that portions of this delta-like domain are conserved among many members of this family of enzymes.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—HMTIIa-M3/6 was constructed by subcloning a 2.5-kb-blighted NotI-XhoI fragment from MTSM-M3/6 (a gift from A. Ashworth (23)) into PvuII/Nhel-cut pMEP4 expression vector (Invitrogen). The M3/6 insert is of full length and contains an amino-terminal myc epitope tag. The resulting expression vector contained a hygroycin B-selectable marker. For mutagenesis purposes, a 1.3-kb NotI fragment from HMTIIa-M3/6 was subcloned into the same sites of pBluescript SKII (Stratagene), which resulted in two NotI sites flanking the M3/6 coding sequence. M3/6 WT, in which M3/6 is placed under the control of the cytomegalovirus promoter, was constructed by subcloning this NotI fragment into the NotI site of pCDNA3 (Invitrogen), which also possesses a selectable marker for neomycin resistance. An hVH-5 construct was a gift of K. Martell (22). pCMV-4HA-SAPK and pCMV-HA-SAPK T183/185YF were gifts from J. Woodgett. The anti-myc epitope antibody 9E10 (Cooper Biomedical) was used for Western blotting, was obtained from the cell culture core, University of Colorado Cancer Center. Anti-myc epitope antibody 9E10 used for immunoprecipitation, and anti-HA epitope 12CA5 were obtained from Babco, Richmond, CA. Antibodies against SAPK and phospho-SAPK (7345 and 6254, respectively) were obtained from Santa Cruz. Antibodies against phospho-ERK1,2 and phospho-p38 (9101 and 9211, respectively) were obtained from New England BioLabs. Phorbol 12-myristate 13-acetate (PMA) and anisomycin were obtained from Sigma. The MEK 1/2 inhibitor PD98059 and p38 MAPK inhibitor SB202474 were purchased from Calbiochem.

Cell Culture and Transfections—K562 cells were cultured in RPMI medium containing 10% fetal calf serum or iron-supplemented bovine calf serum. COS-7 cells were maintained in RPMI containing 10% iron-supplemented bovine calf serum. Transient transfections were performed using either DEAE-dextran or LipofectAMINE-Plus reagent (Life Technologies) according to the manufacturer’s instructions. Stable transfections were performed by electroporation (250 V, 250 μF; Gene Pulser, Bio-Rad Laboratories). One day post-transfection, cells were distributed into 6-well plates in selective media containing 200 μg/ml Hygromycin B (Mediatech; Fisher). After 1 week of selection, individual clones were transferred into 12-well plates for expansion in selective medium.

Western Blot Analysis, Immunoprecipitation, and Metabolic Labeling—Whole cell extracts were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blot analysis was performed as described previously (14). For analysis of inducible M3/6 (myc) protein, K562 cells (107) were metabolically labeled in the absence or presence of 2 μM Cd2+ for 4 h in methionine or phosphate-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed bovine calf serum and 0.1 μCi/ml Tran35S-label (PerkinElmer Life Sciences) or carrier-free 32P3O4 (PerkinElmer Life Sciences). Whole cell extracts were prepared as described previously (14), and myc-tagged M3/6 protein was immunoprecipitated with 9E10 antibody prebound to protein A/G-Sepharose (Calbiochem). Immunoprecipitates were washed extensively in cell lysin buffer, resolved by polyacrylamide gel electrophoresis, and visualized by autoradiography. Scanning densitometry was performed with Alpha Imager 2000 equipment and software.

Site-directed Mutagenesis—Primers were obtained from Life Technologies. Mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with the addition of 1.3 μM betaine (Sigma) using either M3/6 WT or M3/6 subcloned into pBluescript SKII. Mutations were verified by DNA sequencing (DNA Sequencing and Analysis Core, Cancer Center, University of Colorado). Primer sequences are available on request.

Northern Analysis—RNA isolation, transfer, and hybridization conditions were essentially as described previously (24). The full-length hVH5 labeled with [32P]dCTP using the RadPrime kit (Life Technologies) was used as a probe for Northern blots.

RESULTS

PMA Treatment of K562 Cells Induces Increases in the Total Levels of hVH5 mRNA—To examine whether dual-specificity phosphatases, in addition to MKP-1 (14), are regulated during phorbol ester-induced differentiation of K562 cells, we performed Northern analysis of RNA from K562 cells treated with various stimuli. RNA transcripts for hVH5, the human orthologue of M3/6, were previously reported to be most abundant in brain (21); their presence in myeloid tissue was not examined. As shown in Fig. 1A, hVH5 RNA transcripts were not detect-
able in untreated K562 cells but were strongly induced by PMA (which activates the ERK, SAPK, and p38 signal cascades in K562 cells; cf. Fig. 2). To begin to decipher the pathways involved in hVH5 induction, K562 cells were treated with anisomycin, a strong stimulator of the SAPK pathway (25). As with PMA, anisomycin treatment resulted in rapid elevation of hVH5 transcript levels (Fig. 1B), suggesting that activation of the SAPK signal cascade might be responsible for accumulation of hVH5 RNA transcripts. To examine this point further, we performed Northern analysis of RNA from K562 cells treated with anisomycin, in the presence and absence of inhibitors of MEK1/2 (PD98059) and p38 (SB202474). These agents suppress ERK2 and p38 activation, respectively, by over 80% in U937 cells (14). As shown in Fig. 1C, pretreatment with PD98059 or SB202474 did not reduce hVH5 RNA transcript levels in response to PMA treatment, suggesting that activation of the SAPK signal cascade plays a significant role in PMA-induced hVH5 RNA transcript accumulation.

Substrate Specificity of M3/6—To examine substrate specificity of this class of dual-specificity phosphatases in cells, M3/6, the murine orthologue of hVH5, was transfected into K562 cells under the control of an inducible promoter. This construct contained an amino-terminal myc tag recognized by the 9E10(UCCC) antibody, and was driven by the cadmium (Cd^{2+})-inducible human metallothionein promoter (hMTIIa-M3/6). To test substrate specificity of M3/6, equal numbers of cells were incubated at different Cd^{2+} concentrations for 4.5 h, followed by challenge with 200 nM PMA for the last 10 min of incubation to activate the MAPK family of enzymes, SAPK, ERK and p38. Cell lysates were subjected to Western blot analysis to evaluate M3/6 expression, and the extent of phosphorylation of all three protein kinases (26). As shown in Fig. 2A, M3/6 expression was detected at the added Cd^{2+} concentrations of 0.5, 1, and 2 mM, whereas no induction was detected at a Cd^{2+} concentration of 0.1 mM. In the absence of M3/6 expression, PMA treatment induced the phosphorylation of all three protein kinases (Fig. 2, B–D; 0 mM Cd^{2+}). At the higher Cd^{2+} concentrations, SAPK and p38 showed decreased phosphorylation compared with controls with no Cd^{2+} added. Densitometric analysis of the Western blots shown in Fig. 2 indicated that, at 0.5 mM Cd^{2+}, PMA-induced phosphorylation of p38 was reduced by 70%. At the same Cd^{2+} concentration, PMA-induced SAPK phosphorylation was completely prevented; residual phosphorylation was 8% of unstimulated cells. ERK phosphorylation remained unaffected. The same experiments were performed with K562 cells stably transfected with the pMEP4 vector containing no insert. In this case, the levels of PMA-stimulated SAPK and p38 phosphorylation did not differ appreciably from those attained in the absence of Cd^{2+}; the same was true for total protein levels of SAPK (data not shown; cf. also Ref. 14). We also examined levels of phosphorylated SAPK in hMTIIa-M3/6 transfectants treated with Cd^{2+} in the absence of PMA. Cd^{2+} addition alone did not stimulate the phosphorylation of SAPK, and, as expected because of the induction of M3/6, tended to lower background SAPK phosphorylation (data not shown). Thus in K562 cells, M3/6, the orthologue of hVH5, showed more activity toward SAPK than p38 kinase and did not dephosphorylate ERK protein kinases.

Incubation of K562 Cells with PMA Induces the Phosphorylation of M3/6—In PMA-treated cells conditionally expressing M3/6, this protein was detected by the anti-myc 9E10(UCCC) antibody as a broad band (Fig. 2A), suggesting the possibility that PMA treatment resulted in some post-translational modification of the M3/6 protein. To address this point, hMTIIa-M3/6 cells were treated with 2 mM Cd^{2+} for 4 h, followed by PMA treatment for intervals up to 2 h in the continued presence of Cd^{2+}. These cells were then processed for Western analysis. As shown in Fig. 3A, the myc-stained M3/6 band demonstrated a shift toward higher molecular weight that was maximal at 1 h of PMA treatment, decreasing thereafter, with little shift visible by 2 h. To investigate the nature of this upward shift in mobility, hMTIIa-M3/6 cells were treated with Cd^{2+} for 5 h in the presence of [32P]orthophosphate. PMA or anisomycin was added for the last hour of incubation. Following cell lysis, the M3/6 protein was immunoprecipitated with 9E10 antibody, and the washed immunoprecipitates subjected to electrophoresis were followed by autoradiography. The results (Fig. 3B) demonstrate that treatment with PMA markedly increased phosphorylation of the M3/6 protein. Similar results were obtained with anisomycin treatment (Fig. 3B; compare lane 2 with lane 4) in which the increase in phosphorylation was associated with an upward shift in mobility in the M3/6 protein. Thus, PMA and anisomycin induced phosphoryl-

![Fig. 2. Substrate specificity of M3/6 in K562 cells. Equal numbers (10^6) of K562 cells stably transfected with the hMTIIa-M3/6 construct were treated with various concentrations of Cd^{2+} for 4.5 h. Cells were then challenged with 200 nM PMA for 10 min, chilled on ice, and processed for Western analysis. A, M3/6 expression as revealed by staining with the anti-myc antibody 9E10(UCCC). B, the same blot was stripped and reprobed with antibody specific for phosphorylated SAPK; arrow indicates p46 SAPK. C, the blot was stripped and reprobed with antibody specific for phosphorylated ERK1/2, which are not resolved separately here. D, the blot was stripped and reprobed with antibody specific for phosphorylated p38.](image-url)

![Fig. 3. Phosphorylation of M3/6 in K562 cells. A, equal numbers (10^6) of K562 cells stably transfected with the hMTIIa-M3/6 construct were treated with 2 mM Cd^{2+} for 4 h. PMA (200 nM) was added for the indicated intervals, and the cells were processed for Western analysis using the anti-myc 9E10(UCCC) antibody. B, K562 cells stably transfected with the hMTIIa-M3/6 construct were treated with 2 mM CdCl_2 for 5 h in the presence of 0.1 mCi/ml[^32P]O_4. 200 nM PMA (lane 3) or 20 μg/ml anisomycin (lane 4) was added for the last hour of incubation. Immunoprecipitates (9E10 anti-myc antibody) were subjected to electrophoresis followed by autoradiography. Arrows indicate positions of "unshifted" (lane 2) and mobility-shifted (lanes 3 and 4) material at the position of M3/6 protein.](image-url)
Regulation of Dual-specificity Phosphatase M3/6

M3/6 Contains a Delta-like Binding Domain for SAPK—M3/6 and its human orthologue hVH5 contain the amino acid sequence RILPHLYLG at amino acids 163–171 (21, 22), 84 residues amino-terminal to the catalytic consensus sequence. The ILP and L(Y/F)LG elements of this motif occur in all currently sequenced mammalian DSPs at a similar location relative to the active site (Fig. 6A). The presence of the amino-terminal arginine, which is unique to M3/6 and hVH5 among the DSPs, makes this sequence conform to a “delta domain” consensus motif known to be critical for binding of other proteins to SAPK and ERK (29–34; Fig. 6B). Furthermore, it was demonstrated that both the arginine and LXL elements of this motif were important in binding of ERK to the transcription factor Elk-1 (32) and in the interaction of ERK2 with the tyrosine phosphatase PTP-SL (31). We speculated that the delta domain motif might be important in the interaction between M3/6 and SAPK.

To examine whether the delta domain motif affected phosphorylation of M3/6, as inferred from mobility shift, the sequence RILPHLYL was deleted from M3/6. This deleted construct, M3/6Δ, as well as M3/6 C246S and the unmodified M3/6 WT, was transfected into COS-7 cells with HA-SAPK. After 48-h incubation, cell lysates were prepared for Western analysis and the blot was probed with anti-myc 9E10(UCCC) antibody. The results are shown in Fig. 7. Cotransfection into COS-7 cells of HA-SAPK with either wild-type M3/6 or M3/6 C246S resulted in the appearance of a doublet at the position of M3/6 protein, consistent with the induction of phosphorylation. However, cotransfection of HA-SAPK with M3/6Δ resulted in the appearance of only the lower molecular weight band, suggesting that SAPK did not induce the phosphorylation of this protein. Thus, removal of the delta-like domain reduced or prevented the phosphorylation of this protein. The similar degree of presumptive phosphorylation of M3/6 C246S and wild-type M3/6 despite different levels of HA-SAPK expression in this experiment (compare lanes 2 and 3) may reflect the fact that the C246S mutant cannot dephosphorylate SAPK (cf. Fig. 8) and thus might be efficiently phosphorylated at lower levels of SAPK expression than required for phosphorylation of wild-type M3/6.

A transient transfection assay was used to determine the phosphatase activities of wild-type and mutant M3/6 proteins with activated SAPK as a substrate. 48 h after transfection of M3/6 or mutant derivatives and HA-SAPK into COS-7 cells, anisomycin (20 μg/ml) was added to the culture medium for 30 min. Cell lysates were subjected to electrophoresis and Western blot analysis, using the 9E10(UCCC) anti-myc antibody to detect M3/6 expression and an anti-phospho-SAPK antibody to measure the phosphorylation state of SAPK (cf. Fig. 2). The results are shown in Fig. 8. Although wild-type M3/6 reduced anisomycin-stimulated phosphorylation of SAPK, (compare lane 6 with lanes 4 and 2), the mutants M3/6 C246S, M3/6 D215A, and M3/6Δ had no apparent effect on the level of SAPK phosphorylation (Compare lanes 7–9 with lane 6). Densitometric analysis indicated that the band intensities at p54 and p46.
for the three mutants were equivalent to those derived from anisomycin-treated cells transfected only with SAPK or empty vector (lanes 2, 4) and were 2- to 3-fold more intense than those derived from unstimulated cells or cells transfected with wild-type M3/6 (lanes 1, 3, 5, 6). All mutants were equally expressed to the same level as wild-type (data not shown). Thus, removal of the delta-like domain decreased the ability of M3/6 to mediate the dephosphorylation of SAPK to the same extent as a mutation in the essential catalytic cysteine.

Several features of this assay should be emphasized. First, the inclusion of HA-p54-SAPK in the transfection did not markedly increase the level of phosphorylated p54 SAPK fol-
Regulation of Dual-specificity Phosphatase M3/6

**Fig. 7.** Deletion of the M3/6 delta-like domain abolishes SAPK-induced mobility shift. The constructs M3/6WT, M3/6 C246S, and M3/6Δ were cotransfected into COS-7 cells with the construct HA-SAPK wild-type (cf. Fig. 4). The arrow indicates the position of mobility shifted M3/6 (lanes 2, 3). Lower panel: The blot was stripped and reprobed with anti-HA antibody to verify equivalent expression.

**Fig. 8.** Activity of wild-type and mutant M3/6 constructs against activated SAPK. The indicated constructs were transfected into COS-7 cells, and the cells were incubated for 48 h. Where indicated, anisomycin (20 μg/ml) was added for the last 30 min of incubation. Cells were chilled on ice, and cell lysates were prepared for Western analysis with antibody specific for phosphorylated SAPK.

**Fig. 9.** Activity of delta-like domain mutants of M3/6 against activated SAPK. A, amino acid sequence of M3/6 wild-type and delta-like domain mutants. B, activity of R163A mutant. The indicated constructs were transfected into COS cells, and the cells were incubated for 48 h. Where indicated, anisomycin (20 μg/ml) was added for the last 30 min of incubation. Cells were chilled on ice, and cell lysates were prepared for Western analysis with antibody specific for phosphorylated SAPK. C, activity of L168A,L170A mutant. Conditions were as in B above.

**DISCUSSION**

K562 cells are derived from a patient with chronic myelogenous leukemia and can be induced to differentiate along either the megakaryocytic, erythroid, or monocytic lineages (35). It was shown that prolonged PMA stimulation, and specifically prolonged activation of the ERK pathway, led to nonreversible differentiation along the megakaryocyte lineage (1), accompanied by cell flattening, α5β1 integrin expression, and suppression of α-globin expression. In contrast, the effects on morphology and integrin expression were reversible by treatment with the MEK inhibitor PD98059 if PMA treatment was limited to 12 h or less. PMA treatment of K562 cells activates all three major MAPK pathways (Fig. 2). Because the ultimate differentiated phenotype results from integration of the timing, duration, and intensity of signaling from the MAPK pathways, it is clear that a mechanism for highly selective inactivation of one or more of these pathways, in the form of inducible phosphatase activity, could play a role in the differentiation process.

Using Northern blots, we demonstrate that hVH5 transcripts are rapidly induced by anisomycin and PMA, suggesting that hVH5 could play a role in regulating the extent of SAPK/JNK phosphorylation and thus its activity. To test this possibility, we established a K562 cell line with M3/6, the murine orthologue of hVH5, under the control of the metallothionein promoter. We found that Cd2⁺ induction of this protein markedly inhibits the phosphorylation of SAPK/JNK and to a lesser extent p38 protein kinase. To further examine this question, we derived a cell line in which the phosphatase dead mutant of M3/6, C246S, was conditionally expressed under the control of
the hMTIIa promoter. Attempts to derive a cell line constitutively expressing C246S were unsuccessful. In two experiments in which K562 cells expressing the inducible mutant were treated with Cd²⁺ followed by anisomycin or PMA, an increase in SAPK/JNK phosphorylation was observed in the cell line expressing M3/6 C246S (40% in the case of PMA stimulation, data not shown), suggesting that the presence of the C256S dominant negative could prevent dephosphorylation of SAPK. Thus, together these experiments suggest that hVH5 induction plays an important role in regulating SAPK/JNK.

We demonstrate that M3/6 is rapidly phosphorylated following stimulation of K562 cells by PMA or anisomycin and can be directly phosphorylated by SAPK/JNK in transient assays. Brondello et al. (28) recently demonstrated that, in fibroblasts, the DSP MKP-1 was phosphorylated on Ser-359 and Ser-365 by ERK1 and/or ERK2. In contrast to MKP-1, which possesses two potential MAPK phosphorylation sites carboxyl-terminal to the catalytic consensus sequence, M3/6 has 12 potential sites (SP motifs) in this portion of the molecule (21), including one excellent MAPK consensus sequence, a PDSP sequence (amino acids 442–445). We speculate that more than one of these sites is phosphorylated, because broad (Fig. 2) or multiple bands of M3/6 were seen on SDS-polyacrylamide gels after PMA treatment and Western analysis.

Using the proteasome inhibitor LLnL to stabilize MKP-1, cycloheximide to shut off protein synthesis, and an estradiol- and Western analysis, it is phosphorylated, because broad (Fig. 2) or multiple bands of M3/6 (Fig. 3A) were significantly less than the half-life of unphosphorylated MKP-1. Our half-life data, obtained with a pulse-chase approach, in which M3/6 was induced with Cd²⁺ followed by PMA treatment, does not support the idea that phosphorylation leads to the stabilization of M3/6 protein. The half-life of M3/6 protein in the PMA-treated samples was the same as that seen without this treatment. Based on our data, the conclusion that phosphorylation does not prolong half-life appears logical, because 1) the half-life of PMA-induced phosphorylation of M3/6 (Fig. 3A) was significantly less than the half-life of unphosphorylated protein (Fig. 5); 2) the half-life of the retarded band (phosphorylated) in the pulse-chase experiment was identical to the half-life of phosphorylation and did not appear to increase; and 3) only a small percentage (less than 50%) of M3/6 was phosphorylated after PMA treatment, suggesting that phosphorylation could have only a minimal impact on the total levels of M3/6 cellular protein.

Another possible effect of phosphorylation on M3/6 could be to change its location within the cell. It is well known that ERK kinases translocate to the nucleus when phosphorylated (36). M3/6 has been found located in both the nucleus and the cytoplasm (21), depending on cell type. Using immunofluorescent detection of M3/6 transiently transfected into COS-7 cells, we found no difference in M3/6 location with or without treatment with anisomycin, a strong activator of SAPK/JNK. These data suggest that phosphorylation does not markedly change the location of this protein. In addition, we performed localization experiments in which M3/6 C246S was cotransfected into COS-7 cells with SAPK, under the conditions similar to those described in Fig. 8. In this case, expression was also localized to the nucleus in the presence or absence of anisomycin challenge, indicating that phosphorylation of M3/6 did not alter subcellular distribution (data not shown).

SAPK binds to and phosphorylates the transcription factor c-Jun, part of the activator protein-1 transcription factor complex, and thus potentially leads to enhanced transcription of genes with AP-1 sites in their promoters; e.g. c-Fos and c-Jun itself (26, 37). The binding of SAPK to c-Jun is mediated by a short sequence of amino acids termed the delta domain (29, 30, 38). A core region in the delta domain with the consensus sequence (R/K)XXXLX(L/H) has been demonstrated to mediate binding to the SAPK substrate Elk-1 (33). This consensus sequence is also critical for the interaction of ERK2 with the same substrate (32), and a related sequence (RKPDRLVVIP) was shown to target p38 MAPK to transcription factors MEF2A and MEF2C (39). The core sequence is present in other proteins known to be substrates of SAPK and ERK (32–34) and is also critical for the binding of the tyrosine phosphatase PTP-SL to ERK 2 (31). Thus, it is possible that a delta-like domain may be an important element regulating specificity of protein-protein interaction in three of the MAPK cascades.

Interestingly, hVH5 and M3/6 also possess a motif conforming to the consensus sequence of the delta domain core region (Fig. 6, A and B). We have demonstrated that deletion of this motif, RILPHLYL, from M3/6 decreases the ability of SAPK/JNK to phosphorylate this DSP and inhibits the ability of M3/6 to dephosphorylate SAPK/JNK. Furthermore, mutation of the LXL portion of this motif, present in all mammalian DSPs sequenced thus far (cf. Fig. 6A), reduced the ability of M3/6 to dephosphorylate SAPK in COS-7 cells. The amino acid most commonly present at the amino terminus of this motif in other DSPs is aspartic acid (E, Fig. 6A). Unfortunately, we found the R163E mutant protein to be unstable in COS-7 cells, so we could not assess the role of the specific charge at this position on substrate specificity (data not shown). The R163A mutant was not detectably different in activity or specificity from the M3/6 wild-type sequence in our assay. For analogous mutants in the delta-like domain of Elk-1 (32), an LE/LAA mutation was two to three times more effective than an RKAA mutant (the R being 5 residues amino-terminal to LEL in Elk-1, as in the M3/6 motif) in suppressing in vitro phosphorylation by JNK1 and JNK-2; JNK-3 (p54 SAPKβ) was not tested.

Thus, we demonstrate that PMA treatment of K562 cells regulates the levels of hVH5 mRNA. Like the DSP MKP-1, M3/6 is phosphorylated by the protein kinase that it dephosphorylates. We show that M3/6 contains a domain that appears to function similarly to MAPK binding domains found in a diverse set of transcription factors. Our data suggest the interesting possibility that there is competition for binding of MAPK family members between their substrates and the DSPs, which regulate the activity of these enzymes.

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