PB1 Domains of MEKK2 and MEKK3 Interact with the MEK5 PB1 Domain for Activation of the ERK5 Pathway*

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MEKK2 and MEKK3 are MAPK kinase kinases that activate the ERK5 pathway by phosphorylating and activating the MAPK kinase, MEK5. Activated MEK5 then phosphorylates and activates ERK5. PB1 domains were first defined in the $p67^{phox}$ and Bem1p proteins and have been shown to mediate protein-protein heterodimerization. A PB1 domain is encoded within the N-terminal portion of MEKK2, MEKK3, and MEK5. Herein, we analyze the functional role of MEKK2, MEKK3, and MEK5 PB1 domains in the ERK5 activation pathway. The PB1 domains of MEKK2 and MEKK3 bind the PB1 domain of MEK5 but do not significantly homo- or heterodimerize with one another in vitro. Furthermore, co-immunoprecipitation of MEKK2 and MEK5 from cell lysates shows that they form a complex in vivo. Deletion or mutation of the MEKK2 PB1 domain abolishes MEKK2-MEK5 complexes, demonstrating that the PB1 domain interaction is required for MEKK2-MEK5 interactions. Expression in cells of the MEKK2 or MEKK3 PB1 domain inhibits ERK5 activation, whereas expression of a mutant MEKK2 unable to bind the MEK5 PB1 domain or expression of the p67^{phox} PB1 domain has no effect on ERK5 activation. These findings demonstrate that the PB1 domain mediates the association of MEKK2 and MEKK3 with MEK5 and that the respective PB1 domains of these kinases are critical for regulation of the ERK5 pathway. The free PB1 domain of MEKK2 or MEKK3 functions effectively to inhibit the ERK5 pathway but not the p38 or JNK pathways, demonstrating the specific and unique requirement of the MEKK2 and MEKK3 PB1 domain in regulating ERK5 activation.

The <u>phox</u> and <u>Bem1p</u> domain, referred to as the $PB1^1$ domain, functions in mediating protein-protein heterodimerization (1).

The PB1 domain is found in proteins of yeast, plants, and metazoans. The PB1 domain was first identified in p67^{phox}, which is involved in activation of phagocyte NADPH oxidase (2, 3), and Bem1p, the yeast protein functioning in cell polarity (4). Within the sequence of most PB1 domains is encoded the PC motif (phox and Cdc motif) (5), also called OPR (octicosapeptide repeat motif) (6), or AID (atypical PKC interaction motif) (7), which has been recently renamed the OPCA (OPR/PC/AID) motif (1). OPCA motifs are encoded in the C-terminal region of most PB1 domains. PB1 domains have been shown to heterodimerize using a β grasp topology in a "front-to-back" arrangement (8). Acidic clusters of amino acids in the OPCA motif interact electrostatically with basic clusters in the PB1 domain. Of the mouse and human PB1 domain-encoding proteins, five are serine-threonine kinases: MEKK2, MEKK3, MEK5, and two atypical protein kinase C (aPKC) enzymes. PKC λ and PKC ζ .

MEK5 is the MAPK kinase that phosphorylates and activates ERK5 in response to growth factors, oxidative stress, and hyperosmotic conditions (9-14). MEKK2 and MEKK3 are MAPK kinase kinases that bind, phosphorylate and activate MEK5 (15, 16). MEKK2 and MEKK3 encode PB1 domains within their N-terminal sequences (8, 17). Our previous studies using yeast two-hybrid analysis had shown that the N terminus of MEKK2 is required for its binding to MEK5 (15). The PB1 domain of MEKK2 is encoded within the N terminus of MEKK2, suggesting that the PB1 domain contributes to MEKK2 interaction with MEK5. Therefore, we hypothesized that the PB1 domains were involved in the interaction of MEKK2 and MEKK3 with MEK5. Herein, we demonstrate that the PB1 domain of MEKK2 or MEKK3 specifically interacts with the PB1 domain of MEK5. Furthermore, expression of the MEKK2 or MEKK3 PB1 domain in cells inhibits ERK5 activation. Taken together, our findings show that the PB1 domain confers specificity in the interaction of MEKK2 and MEKK3 with MEK5 and that an isolated MEKK2 or MEKK3 PB1 domain behaves as an inhibitor of the ERK5 pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Wild type and MEKK2–/– mouse embryonic fibroblasts (MEFs) were isolated as described previously (18).

DNA Constructs—The cDNAs for the conserved PB1 domains of MEKK2 (amino acids (aa) 35–127), MEKK3 (aa 36–128), MEK5 (aa 10–102), and p67^{phox} (aa 342–433) were amplified using PCR and ligated into pGEX, pRSET, and pMSCVpuro vectors. For the pMSCV-puro constructs, a FLAG tag was inserted upstream of the PB1 domain coding sequence. For the MEKK2 PB1 domain mutation constructs, amino acid residue lysine (Lys) 47 was replaced by alanine (Ala) using a site directed mutagenesis. Wild type, Lys to Ala mutant (K-A MEKK2), and PB1 domain deleted MEKK2 (Δ PB1 MEKK2) (aa 123–619) were subcloned into the pMSCVpuro vector. FLAG-MEK5, HA-MEKK2, and HA-MEKK3 constructs were described previously (15).

Generation of Cell Lines Expressing FLAG-PB1 Domains and MEKK2 Proteins—Phoenix cells were used as packaging cells for the retroviral gene transfer system (19). pMSCVpuro vectors were transfected into Phoenix cells using Lipofectamine Plus transfection reagent (Invitrogen). Two days after transfection, the supernatants were collected and diluted with the same volume of fresh complete IMDM, and polybrene was added to the infection medium at 8 μ g/ml. The infection mixtures were added to MEF cultures seeded on 6-cm dishes, incubated overnight, and then replaced with fresh complete IMDM containing 50

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¹ The abbreviation used are: PB1, *phox* and Bem1p; OPCA, OPR, PC, and AID; aPKC, atypical protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEFs, mouse embryonic fibroblasts; HA, hemagglutinin; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK ki-

nase; MEKK, MEK kinase; IMDM, Iscove's modified Dulbecco's medium; Ab, antibody; mAb, monoclonal Ab; aa, amino acids.

 $\mu g/ml$ puromycin. The puromycin-resistant cells were isolated and propagated for use in the experiments.

RESULTS

PB1 Domains of MEKK2, MEKK3, and MEK5—Previously, we demonstrated in a yeast two-hybrid screening that the N terminus of MEKK2 encoding amino acids 1-188 bound to MEK5 (15). Furthermore, truncation of the first 125 amino acids of MEKK2 resulted in loss of MEK5 binding. The PB1 domain of MEKK2 is encoded within amino acids 43-122, consistent with this region of MEKK2 being important for the interaction between MEKK2 and MEK5. Fig. 1A shows the sequence alignment of the MEKK2, MEKK3, MEK5, p67^{phox} PB1 domains compared with yeast Bem1p PB1 sequence. Although the PB1 domain structure is highly similar to the Ras binding domain of c-Raf1 (4), PB1 domains have not been found to bind Ras proteins. PB1 dimers have recently been described as having a β grasp topology with a front-to-back binding arrangement (8). The OPCA motif is a conserved sequence in the C-terminal moiety of the PB1 domain (1). It is worth noting that MEKK2 and MEKK3 have rather weak consensus OPCA motifs. Acidic clusters of amino acids in the OPCA motif of one PB1 domain bind to basic amino acids including the conserved Lys residue in the N-terminal moiety of a different PB1 domain. Mutation of the conserved Lys residue in Bem1p and p67^{phox} was shown to completely disrupt its binding to Cdc24p and $p40^{phox}$, respectively (2). The defined function of PB1 domains appears to compose heterodimerization of the cognate proteins.

To characterize the properties of the MEKK2, MEKK3, and MEK5 PB1 domains, we expressed each PB1 domain as a GST-fusion and His/Xpress-tagged recombinant protein (Fig. 1, *B-D*). The $p67^{phox}$ PB1 domain was also expressed as a control for binding studies presented below. In addition, to see the role of the conserved Lys residue, a MEKK2 PB1 domain construct having Ala substituted for Lys⁴⁷ was also prepared. The recombinant PB1 domain proteins were then used to define their ability to form hetero- or homodimeric complexes using pull-down assays and immunoblotting (Fig. 1, B and C). The MEKK2 PB1 domain bound MEK5. However, the association seemed less stable when the MEKK2 PB1 domain was expressed as a His/ Xpress-tagged protein and MEK5 PB1 domain as a GST fusion than the reciprocal expression of the fusion proteins (compare Fig. 1, *B* and *C*). The reason for this difference is unclear but may be related to some differential interfering effect of GST and His/Express tag portions on PB1 domain function. Importantly, the K-A MEKK2 mutant PB1 domain or the p67^{phox} PB1 domain did not bind to MEK5. The MEKK3 PB1 domain also bound the MEK5 but not the p67^{phox} or MEKK2 PB1 domain. Cumulatively, these findings indicate a selective interaction of the MEKK2 and MEKK3 PB1 domains with the MEK5 PB1 domain.

Pull-down of MEK5 from Lysates of Mouse Embryo Fibroblasts-Fig. 2A shows that the PB1 domains of MEKK2 and MEKK3 bind to endogenous MEK5 from lysates of wild type MEFs. The p67^{phox}, K-A MEKK2 mutant, and MEK5 PB1 domains failed to bind and pull-down MEK5 from MEF lysates. To verify the binding of endogenous MEK5 to MEKK2 and MEKK3 PB1 domain, a FLAG-tagged MEK5 was expressed in MEFs (Fig. 2B). Similar to endogenous MEK5, expressed MEK5 could be selectively pulled down by the MEKK2 and MEKK3 PB1 domains but not the p67^{phox}, K-A MEKK2 mutant, or MEK5 PB1 domain. Similarly when MEKK2 or MEKK3 were expressed in cells, the MEK5 PB1 domain was able to pull down MEKK2 (Fig. 2C) and MEKK3 (Fig. 2D). These data clearly demonstrate that in mammalian cells the MEKK2 and MEKK3 PB1 domains selectively bind to MEK5 and vice versa, the MEK5 PB1 domain selectively binds MEKK2 and MEKK3. The PB1 domains do not appear to



FIG. 1. Interaction of MEKK2, MEKK3, and MEK5 PB1 domains in vitro. A, sequence alignment of the mouse MEKK2, MEKK3, MEK5, and p67^{phox} PB1 domains and OPCA motifs with yeast Bem1p sequence. The conserved lysine in PB1 domain is in gray. This Lys residue is substituted with Ala in the K-A MEKK2 mutant. Shading indicates conserved OPCA sequence (h, hydrophobic amino acid). Consensus PB1 domain sequence is based on an alignment of the known PB1 domain-containing proteins. B and C, GST pull-down assays to measure the in vitro binding of GST fusions of the PB1 domains of MEKK2, K-A MEKK2, MEKK3, MEK5, and p67^{phox} with His/Xpresstagged PB1 domains of the same proteins. Pull-down assays were conducted with the indicated GST-PB1 domain fusion protein and His/ Xpress-tagged protein. The adsorbents were immunoblotted with anti-Xpress Ab. D, anti-Xpress Ab blot of 1% of the total His/Xpress-tagged protein used for each pull-down assay, demonstrating similar expression of each protein.

significantly bind to themselves (homodimeric interactions). The modest pull-down of HA-MEKK3 with the MEKK3/ MEKK2 PB1 domains in Fig. 2D may suggest that an *in vivo*



FIG. 2. Binding of full-length MEK5, MEKK2, and MEKK3 by the PB1 domains of MEKK2, MEKK3, and MEK5. GST-PB1 domain fusion proteins were used for the pull down assays from MEF lysates. *A*, immunoblot of endogenous MEK5 pulled down from MEF lysates. *B*, immunoblot of FLAG-MEK5 pulled down from FLAG-MEK5 transfected MEF lysates. *C*, immunoblot of HA-MEKK2 pulled down from HA-MEKK2 transfected MEF lysates. *D*, immunoblot of HA-MEKK3 pulled down from HA-MEKK3 transfected MEFs. For each pull-down assay 1 mg of total MEF lysate was used for *A*-*D*.

complex of MEK5 and MEKK3/MEKK2 proteins exists in cells, since bacterially expressed, recombinant MEKK3 PB1 domain did not bind itself, MEKK2, or p67^{phox} PB1 domains (Fig. 1).

MEKK2 PB1 Domain Is Required for Co-immunoprecipitation of MEKK2 and MEK5—MEKK2–/– MEFs (18) were used to add back wild type MEKK2, K47A mutated MEKK2 (K-A MEKK2), or a MEKK2 protein whose PB1 domain had been deleted (Δ PB1 MEKK2). A MEKK2 monoclonal antibody was then used to immunoprecipitate the different MEKK2 proteins. Fig. 3 shows that only the wild type MEKK2 protein was capable of constitutively associating with MEK5 for co-immunoprecipitation. Neither the K-A mutant that disrupts PB1 domain binding nor the Δ PB1 MEKK2 protein bound MEK5. These findings indicate that the MEKK2 PB1 domain is required for stable association of MEKK2 and MEK5.

MEKK2 and MEKK3 PB1 Domains Bind MEK5 in Vivo and Inhibit ERK5 Activity—To examine the functional consequence of expressing the free PB1 domains of MEKK2, MEKK3, and MEK5 on the ERK5 signaling pathway, FLAG-tagged MEKK2, K-A MEKK2, MEKK3, and p67^{phox} PB1 domains were expressed in MEFs. Unfortunately, we were unable to express the FLAG-tagged MEK5 PB1 domain in MEFs (data not shown). The MEKK2, K-A MEKK2, MEKK3, and p67^{phox} FLAG-tagged proteins were efficiently expressed in MEFs. FLAG-MEKK2 and FLAG-MEKK3 PB1 domains bound endogenous MEK5 as shown by co-immunoprecipitation (Fig. 4A). In contrast, neither the mutant K-A MEKK2 nor p67^{phox} PB1 domain interacted with MEK5 (Fig. 4A). Total cell lysates prepared from the different FLAG-PB1 domain expressing cells were used to measure ERK5 activity. The level of ERK5 activity was assessed by a mobility shift of the ERK5 band in the gels (Fig. 4B). The slower migrating band of the ERK5 doublet has been shown to be caused by ERK5 phosphorylation that correlates with its activation (16, 20, 21). In the presence of 10% serum, ERK5 activation was readily observed in MEFs. Neither FLAG-K-A MEKK2 nor FLAG-p67^{phox} PB1 domain showed any inhibitory activity toward the ERK5 mobility shift. In contrast, both FLAG-MEKK2 and FLAG-MEKK3 PB1 domains completely blocked ERK5 activation.

FLAG-MEKK2 and FLAG-K-A MEKK2 PB1 domain expressing cells were further analyzed with sorbitol stimulation, a hyperosmotic stress that activates ERK5 in a MEKK2-dependent manner (14) (Fig. 4*C*). Sorbitol induced an ERK5 mo-



FIG. 3. **MEKK2 PB1 domain interaction with MEK5** *in vivo*. Wild type (*WT*), K-A MEKK2, and Δ PB1 MEKK2 were expressed in MEKK2–/– MEFs using retroviral transfection. MEKK2 protein was immunoprecipitated from the cells using an anti-MEKK2 mAb. The immunocomplexes were analyzed by anti-MEK5 blotting.

bility shift in control and FLAG-p67^{phox} PB1 domain expressing MEFs. This mobility shift was abolished by expression of a FLAG-MEKK2 PB1 domain, but not by expression of the FLAG-K-A MEKK2 PB1 domain. Cumulatively, these data indicate that an expressed MEKK2 or MEKK3 PB1 domain binds to endogenous MEK5 and induces a refractory state of the MEKK2 or MEKK3/MEK5/ERK5 signaling pathway so that ERK5 is not activated. Importantly, sorbitol activation of p38 MAPK or JNK was not inhibited by expression of the MEKK2 PB1 domain (Fig. 4*C*). Thus, the inhibitory effect of the MEKK2 PB1 domain is specific for the ERK5 pathway.

DISCUSSION

MEKK2 and MEKK3 are unique among the MAPK kinase kinases in their ability to regulate the ERK5 pathway (15). No other MAPK kinase kinase has been shown to regulate MEK5 and activation of the ERK5 pathway. Interestingly, it has been reported that PKC ζ phosphorylates MEK5 and activates the ERK5 pathway (22), although this result has been recently disputed (17). Our work indicates that the specificity of MEKK2 and MEKK3 regulation of the ERK5 pathway resides in the PB1 domains encoded in the N-terminal region of MEKK2, MEKK3, and MEK5. The PB1 domain of MEKK2 or MEKK3 specifically heterodimerize with the PB1 domain of MEK5. A functional PB1 domain is absolutely necessary for interaction of full-length MEKK2 with endogenous MEK5. Mutation of the conserved Lys residue, which inhibits PB1 domain binding, or deletion of the PB1 domain in MEKK2 abolishes MEKK2 binding to MEK5. Furthermore, a free MEKK2 or MEKK3 PB1 domain inhibits ERK5 activation in response to serum or hyperosmotic stress. This result is striking and strongly suggests that the PB1 domain interaction of MEKK2 or MEKK3 with MEK5 is required for MEK5 activation of ERK5. Disruption of the MEKK2 and MEKK3 interaction with MEK5 using the free PB1 domains appears to be sufficient to inhibit the ERK5 pathway. It should be noted that when we overexpressed wild type MEKK2, K-A MEKK2, or $\Delta PB1$ MEKK2, ERK5 became activated (data not shown). This indicates that overexpression of MEKK2 proteins even lacking a functional PB1 domain could still activate the ERK5 pathway, suggesting that the overexpression of these proteins predictably bypasses the need for normal assembly of a regulatory complex. It is clear from our previous studies that MEKK2 expression is required for the activation of the ERK5 pathway by growth factors and oxidative and hyperosmotic stresses (14, 15). The targeted gene disruption of MEKK2 abolishes ERK5 activation to these stimuli (14), and we now demonstrate that the free MEKK2 or MEKK3 PB1 domain inhibits endogenous ERK5 activation.

Our studies with MEKK2-/- MEFs, where we created a MEKK2 knockout by targeted disruption of the MEKK2 gene, has shown that MEKK2 coordinately regulates the ERK5 and JNK pathways (14). MKK7, the MAPK kinase that regulates JNK, has been characterized to bind to the C-terminal region of

FIG. 4. MEKK2 and MEKK3 FLAG-PB1 domains interact with MEK5 and inhibit ERK5 activation. FLAG-tagged MEKK2, K-A MEKK2, MEKK3, or p67^{phox} PB1 domains was expressed in MEFs. A, anti-MEK5 immunoblot of FLAG Ab immunoprecipitates from cells expressing the indicated FLAG-tagged PB1 domains. Left and right panels show anti-MEK5 Ab and FLAG Ab immunoblots, respectively. B, ERK5 mobility shift assay of the indicated PB1 domain expressing MEFs grown in 10% serum. C, FLAG-PB1 domain expressing MEFs were serum starved for 12 h and then stimulated with 0.2 M sorbitol for 20 min, and the ERK5 mobility shift was analyzed by anti-ERK5 Ab immunoblotting. The activation of p38 and JNK was analyzed by immunoblotting with phospho-specific antibodies for p38 and JNK, respectively

MEKK2 (20, 23), whereas MEK5 binds to the N-terminal PB1 domain of MEKK2. Thus, the MAPK kinases for the JNK and ERK5 pathways bind different regions of MEKK2. Loss of MEKK2 expression in mouse embryo fibroblasts results in loss of ERK5 activation in response to growth factors such as FGF-2 and both oxidative and hyperosmotic stresses (18). Normal JNK activation in the presence of free MEKK2 PB1 domain demonstrates that MEK5 PB1 domain interaction with the PB1 domain of MEKK2, MEKK3, or other proteins is not needed for the MEKK2-mediated regulation of JNK activation. Similarly, expression of MEKK2 or MEKK3 PB1 domains has no inhibitory effect on p38 activation, consistent with the PB1

domain of MEKK2, MEKK3, and MEK5 functioning in the regulation of ERK5, but not JNK or p38 activation.

During the course of these studies, Lamark et al. (17) reported that MEK5 bound to the adapter protein, p62, via PB1-PB1 domain interactions. The p62 adapter protein has been shown to be important in forming a complex with TrkA, the neurotrophic receptor tyrosine kinase, and to be critical for neurotrophin activation of the ERK5 pathway (24). It has been proposed that p62 is critical for forming a signaling complex in endosomes that also includes the aPKCs (25) and that these responses are critical for neuronal differentiation (24, 26). It is presently unclear if MEKK2 or MEKK3 is associated with the p62-aPKC-MEK5 complex. Certainly, a model could easily be envisioned where PB1 domains of p62, MEK5, MEKK2, and/or MEKK3 along with the aPKCs might form an oligomeric signaling complex.

In conclusion, our results, for the first time, show the specific interaction of the MEKK2 and MEKK3 PB1 domains with the MEK5 PB1 domain. Expression of the MEKK2 or MEKK3 PB1 domain selectively inhibited ERK5 but not JNK or p38 activation. The fact that mutation of the conserved Lys to Ala in the MEKK2 and MEKK3 PB1 domains inhibited interaction with the MEK5 PB1 domain, and resulted in the failure to inhibit ERK5 activation in cells, is consistent with very specific sequence requirements that are necessary for the selective PB1-OPCA interactions we and others (8, 17) have observed.

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