The N-terminal ERK-binding Site of MEK1 Is Required for Efficient Feedback Phosphorylation by ERK2 in Vitro and ERK Activation in Vivo*

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An ERK2-binding site at the N terminus of MEK1 was reported to mediate their stable association. We examined the importance of this binding site in the feedback phosphorylation of MEK1 on Thr292 and Thr386 by ERK2, the phosphorylation and activation of ERK2 by MEK1, and the interaction of MEK1 with ERK2 and Raf-1. Deletion of the binding site from MEK1 reduced its phosphorylation by ERK2, but had no effect on its phosphorylation by p21-activated protein kinase-1 (PAK1). A MEK1 N-terminal peptide containing the binding site inhibited MEK1 phosphorylation by ERK2. However, it did not affect MEK1 phosphorylation by p21-activated protein kinase or myelin basic protein phosphorylation by ERK2. Deletion of the N-terminal ERK-binding domain of MEK1 also reduced its ability to phosphorylate ERK2 in vitro, to co-immunoprecipitate with ERK2, and to stimulate ERK2 activation in transfected cells, but it did not alter the association with endogenous Raf-1. Using ERK2-p38 chimeras and an ERK2 deletion mutant, a MEK1-binding site of ERK2 was localized to its N terminus.

The MAP1 kinases ERK1 and ERK2 are activated by a protein kinase cascade under the control of the small G protein Ras. Ras binds to Raf with high affinity and causes Raf translocation to the membrane, where it is activated by mechanisms including phosphorylation of serine and tyrosine residues and regulated interactions with other proteins (1). Raf activates MEK1 and MEK2, the dual-specificity protein kinases that directly phosphorylate and activate ERK1 and ERK2 (2, 3). Reconstitution experiments with purified proteins in vitro demonstrated that these MEK proteins are sufficient to activate ERK proteins (4). Mansour et al. (5) and others identified mutations that created constitutively active forms of MEK1 and MEK2. When introduced into mammalian cells, these activated MEK mutants increase ERK activity and induce effects associated with activation of the Ras/ERK pathway such as transformation of fibroblasts, development of tumors in nude mice, and outgrowth of neurites in PC12 cells (5–7).

The multienzyme ERK cascade allows for amplification because MEK1 and MEK2 are present in considerable excess of Raf. However, the MEK and ERK proteins from this pathway are present at roughly equal concentrations and, in some cells, MEK proteins are in excess (8). This suggests that the MEK to ERK step exists not for amplification, but for kinetic regulation and to receive additional modulatory inputs (8–10). The intermediate step introduces mechanisms for sensing coincident and distinct regulatory inputs (11).

In the budding yeast pheromone response pathway, the MAP kinase cascade is organized on a scaffold Ste5p (12, 13). No functional homolog of Ste5p has yet been identified for the mammalian ERK pathway, although the recently discovered protein MP-1 has been reported to enhance interactions between MEK1 and ERK1 and thereby increase signaling through the pathway (10). Specialized domains within the kinases themselves may also facilitate productive interactions among components of the cascade. MEK1 and MEK2 contain a unique proline-rich insert not present in any other MEK family members. Deletion of this insert reduces the ability of MEK to cause ERK activation in transfected cells, although loss of the insert has little effect on the inherent enzymatic activity of MEK, and the insert does not directly bind to ERK2 (9, 14). MEK1 contains two sites of phosphorylation for ERK2 (15–17). One site is near the MEK C terminus, and the second is in the proline-rich insert. These sites are phosphorylated in intact cells (16). In vitro and in cells, the proline-rich insert of MEK1 is also phosphorylated by the p21-activated kinase PAK1, among others (11). These findings suggested that the regulated binding of the insert to an unknown factor improved the efficiency of ERK activation.

A binding site for ERK2 at the N terminus of MEK1 was found to mediate their stable association (18). This type of interaction may also take place between other MEK/MAP kinase pairs. Because MEK1 contains a nuclear export sequence, it has been suggested that the binding to MEK1 retains ERK2 in the cytosol of unstimulated cells (18). We examined the impact of this binding site on the feedback phosphorylation of MEK1 on Thr292 and Thr386 by ERK2 and the activation of ERK2 by MEK1. Deletion of the binding site reduced the rate of phosphorylation of MEK1 by ERK2, but had no effect on MEK1 phosphorylation by PAK1. Deletion of the N terminus of MEK1 also reduced its ability to phosphorylate ERK2 in vitro and to stimulate ERK1 and ERK2 in transfected cells. Using ERK2-p38 chimeras and an ERK2 deletion mutant, a site of MEK1 binding was localized to the N terminus of ERK2. This study implies a significant role for the ERK-MEK noncatalytic association in signaling through the cascade.

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The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; PAK, p21-activated kinase; GST, glutathione S-transferase; EGF, epidermal growth factor; MBP, myelin basic protein.
MATERIALS AND METHODS

Subcloning, Mutagenesis, and Protein Expression—The pRSET-MEK1(FL) and pRSET-MEK1(ΔN1) constructs were kindly provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO). ΔN1 lacks the first 32 amino acids of MEK1 (19). To create kinase-dead versions of these constructs, the conserved lysine 97 in subdomain II was mutated to methionine using the QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's directions. The oligonucleotides used for mutagenesis were 5'-GGTCAATGCGCAGAATGCTAATCCATCTGG-3' and 5'-CCAGATTAGTACCATCTGGCCATGAC-3'. To subclone full-length MEK1 and MEK1-ΔN1 DNAs into pcMV5-Myc, the DNAs were digested with HindIII first, filled in by Klenow, and then digested with BamHI. 1.5-kilobase pair DNA fragments were gel-isolated and ligated into pcMV5-Myc digested with BamHI-SmaI. pCPEHA-ERK2 was as described (11), and pcMV5-Erk1-His6 was kindly provided by Peter Shaw.

His-tagged, kinase-dead MEK1 proteins were expressed and purified in Escherichia coli strain BL21(DE3). Overnight cultures grown in terrific broth containing 25 μg/ml chloramphenicol and 125 μg/ml ampicillin were inoculated into 1 liter of TB/chloramphenicol/ampicillin. The culture was grown at 30 °C until OD600 = 0.4–0.6 and induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside overnight at room temperature. Cell pellets were snap-frozen in liquid nitrogen, thawed on ice, and resuspended in sonication buffer (50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and protease inhibitors). Cells were lysed in a glass homogenizer and then sonicated. Supernatants were adjusted to 1% Tween 20, 1 mM NaCl, and 20% glycerol, and MEK proteins were purified as described (15). The concentrations of MEK proteins were estimated by comparison of serial dilutions to bovine serum albumin standards on the same gel. MEK1-ΔN1 and full-length MEK1 express equally well in bacteria and in mammalian cells (see below).

Active ERK2 was prepared and purified as described (20). GST-PAK1-232–544 was kindly provided by Jeff Frost (University of Texas Southwestern Medical Center). His6-tagged wild-type MEK1, MEK1-T292A, and MEK1-T386A proteins were kindly provided by Dr. Ahn. Recombinant GST-ERK2-K52R was expressed and purified as described (4). His-tagged ERK2-p58 chimeras were created and purified as described (21). GST-ERK2 truncated after ERK2 residue 337 was kindly provided by Megan Robinson. The two MEK1 N-terminal peptides synthesized were P1 (KKKPTQQLNPPAGSASVNG) and P2 (RRRLNPAGASAVNGTSSAE), which overlaps with P1 as indicated by underlining. Neither was a substrate for ERK2.

Kinase Assays—Kinase reactions were carried out in 30 μl of 10 mM Hepes (pH 8.0), 10 mM MgCl2, 1 mM benzamidine, 1 mM dithiorthiole, and 50 μM ATP (1 pmol/fmol) at 30 °C for the indicated times. Reactions were stopped by adding 6 μl of 5× SDS sample buffer, followed by boiling for 2 min.

Cell Culture, Transfection, and Preparation of Extracts—Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium and maintained for 24 h prior to harvest. For immunoprecipitation kinase assays, cells from each 60-mm dish were collected and lysed in 0.5 ml of 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 0.2 mM Na2VO4, and protease inhibitors. For co-immunoprecipitation, cells were lysed in a Dounce apparatus in 0.25 ml of lysis buffer containing no detergent. As indicated, cells were stimulated with 100 ng/ml EGF for 5 min.

Antibodies and Immunoblotting—Antibodies were obtained from the following sources: monoclonal antibody to the Myc epitope (9E10), Cell Culture Center; monoclonal antibody to the hemagglutinin epitope (12CA5), Berkeley Antibody Co.; anti-ERK1 antibody Y691, raised as described (22); anti- phospho-ERK antibodies, Promega and New England Biolabs Inc.; anti-Raf-1 antibody (SC-133), Santa Cruz Biotechnology; and anti-Erk1 antibody, New England Biolabs Inc. To detect overexpressed or endogenous proteins, ~20 μg of cell lysates were loaded on an SDS-polyacrylamide gel and electrophoresed onto nitrocellulose paper. Blots were developed using enhanced chemiluminescence.

Immunoprecipitation—Proteins precipitated from 0.2 ml of cell lysates with 2 μl of antibody and 30 μl of protein A-Sepharose beads were used for immune complex kinase assays. Beads were washed three times with 20 mM Tris-HCl (pH 7.4) and 1 mM NaCl and once with 10 mM Hepes (pH 8.0), and 10 mM MgCl2.

RESULTS

The N-terminal ERK-binding Domain of MEK1 Is Required for Its Efficient Feedback Phosphorylation by ERK2—Several substrates of the MAP kinase ERK2, including the ternary complex factor Elk-1, contain a targeting domain (D domain) for ERK2 that is required for their efficient phosphorylation and activation (23, 24). The MAP kinase MEK1 is the physiological upstream enzyme for ERK2. MEK1 binds to ERK2 via its N-terminal 32 amino acids (18). Within the MEK1 N terminus, there is a sequence that resembles a D domain motif. Because MEK1 is also a substrate for ERK2 both in vitro and in vivo (15–17), we asked if deletion of the ERK-binding domain of MEK1 affected its phosphorylation by ERK2.

His-tagged, bacterially expressed, kinase-dead full-length MEK1 and an N-terminal deletion mutant (MEK1-ΔN1) lacking residues 1–32 were used as substrates for active ERK2 (Fig. 1A). In the time course of Fig. 2A, phosphorylation of the N-terminal deletion mutant ΔN1 by ERK2 was much reduced compared with that of full-length MEK1; after 80 min, maximal phosphorylation of ΔN1 was only ∼25% of that of full-length MEK1. When increasing concentrations of MEK1 proteins from 10 to 350 μM were used in the kinase reactions, phosphorylation of full-length MEK1 by 16 nm ERK2 was greater than that of ΔN1 at equivalent concentrations (Fig. 2B). The data suggest that phosphorylation of ΔN1 is still in the linear range even at 350 μM. These results indicate that the ERK-binding domain enhances the rate of feedback phosphorylation of MEK1 by ERK2.

The p21-activated protein kinase PAK1 phosphorylates MEK1 on Ser328 in vitro and in vivo (11). To show that the ERK-binding domain is not required for MEK1 phosphorylation by other protein kinases, constitutively active, recombinant PAK1 was used to phosphorylate full-length MEK1 and ΔN1 in vitro (Fig. 2C). Whereas ERK2 phosphorylated MEK1 to a higher extent than ΔN1 over the time course examined, PAK1 phosphorylated ΔN1 MEK1 at least as well as full-length MEK1. This indicates that deletion of the ERK-binding domain from MEK1 does not impair its phosphorylation by other protein kinases.
FIG. 2. Deletion of the N-terminal ERK-binding domain of MEK1 reduces its phosphorylation by ERK2.
A, time course of phosphorylation of full-length MEK1-K97M (FL) and MEK1-ΔN1 K97M by active ERK2. MEK1 proteins (180 nM) were phosphorylated by 16 nM active ERK2 for the indicated times. Upper panel, autoradiogram; lower panel, plot of incorporation of 32P into MEK1 proteins determined by scintillation counting. Data are shown as relative stoichiometry. One of five similar experiments is shown.

B, concentration dependence of MEK1-K97M and MEK-1ΔN1 K97M phosphorylation by ERK2. The indicated concentrations of MEK1 proteins were phosphorylated by 16 nM active ERK2 for 30 min. Upper panel, autoradiogram; lower panel, plotted as percent of maximal incorporation. One of three similar experiments is shown.

C, phosphorylation of MEK1-K97M proteins by active ERK2 and GST-PAK1-(232–544). MEK1 proteins (350 nM) were phosphorylated by 16 nM active ERK2 or 750 ng of PAK1-(232–544) for the indicated times. Upper panel, autoradiogram; lower panel, plotted as relative stoichiometry. One of two similar experiments is shown. The increase in phosphorylation of ΔN1 by PAK at 60 min was observed in both experiments. Its significance is unknown.
A Peptide Containing the ERK-binding Domain of MEK1 Inhibits MEK1 Phosphorylation by ERK2—To gain further evidence that the ERK-binding domain of MEK1 is required for its efficient phosphorylation by ERK2, two MEK1 N-terminal peptides were used as competitors in the kinase assays. Peptide P1 (residues 3–22) contains the ERK-binding domain, whereas the control peptide P2, which contains three N-terminal arginines linked to residues 11–27 of MEK1, is C-terminal to the binding domain, but overlaps P1 within the deleted region (Fig. 1F). As expected, P1 inhibited phosphorylation of MEK1 by ERK2 in a concentration-dependent manner (Fig. 3A). Consistent with these results, P1 has little effect on phosphorylation of MEK1-ΔN1 by ERK2 (data not shown). Significant inhibition of phosphorylation of full-length MEK1 by ERK2 was observed with as little as 20 μM P1. On the other hand, P2 had little effect on MEK1 phosphorylation by ERK2 even at 200 μM. This result shows that a peptide containing the ERK-binding domain itself can efficiently inhibit phosphorylation of MEK1 by ERK2, suggesting that the ERK-binding domain can compete with MEK1 for binding to ERK2.

To demonstrate the specificity of inhibition, the ability of the peptide to block MEK1 phosphorylation by PAK1 was tested (Fig. 3B). Whereas clear inhibition was observed for phosphorylation by ERK2, little or no inhibition was observed with PAK1 even with 100 μM P1. This supports the conclusion that the effect of P1 is not on MEK1 itself, but on its interaction with ERK2. To test whether P1 can inhibit ERK2 activity toward other substrates, the effect of P1 on phosphorylation of MBP and the ternary complex factor Elk-1 by ERK2 was assessed (Fig. 3C). Even 200 μM P1 had little effect on phosphorylation of MBP by ERK2. This indicates that P1 is not a general inhibitor of ERK2, independent of substrate. On the other hand, phosphorylation of Elk-1, which is targeted to ERK2 by a specific MEK1-interacting domain, was also inhibited partially at 200 μM, the highest concentration of P1 tested.

Phosphorylation of MEK1 on Thr292 and Thr386 by ERK2 Is Inhibited by P1—ERK2 phosphorylates Thr292 and Thr386 by ERK2, which is located within the MEK1 proline-rich insert, and Thr386, which is near its C terminus. P1 at 100 μM reduced MEK1 phosphorylation by ERK2 to ~25% of that without P1. To investigate whether phosphorylation of both Thr292 and Thr386 is inhibited by P1, we used the T292A and T386A mutants as substrates for ERK2 in the presence or absence of 100 μM P1 (Fig. 4). P1 strongly inhibited ERK2 phosphorylation of both residues of MEK1 because MEK1-T386A and MEK1-T292A were each phosphorylated less well in the presence of 100 μM P1.

Deletion of the ERK-binding Domain from MEK1 Reduces Its Phosphorylation of ERK2 in Vivo and Activation of Endogenous ERK1/2 in Vivo—Thus far, we have shown that the MEK1 N-terminal ERK-binding domain is required for the efficient phosphorylation of MEK1 by ERK2. Because ERK2 is a physiological substrate of MEK1, we wanted to test whether deletion of the ERK2-binding domain of MEK1 would diminish its phosphorylation and activation of ERK2. Equal amounts of overexpressed, Myc-tagged full-length MEK1 and MEK1–ΔN1 were immunoprecipitated from 293 cells and used in vitro to phosphorylate GST-ERK2-K52R (Fig. 5, A and B). Wild-type MEK1 had significantly higher activity toward ERK2 than did ΔN1 isolated from either unstimulated or EGF-stimulated cells. We considered the possibility that deleting residues 1–32 of MEK1 might reduce its intrinsic activity toward ERK2, thereby decreasing the extent of its activation (19). This is unlikely to contribute significantly to reduced ERK activation because, as shown in Fig. 7, ΔN1 phosphorylated a protein lacking a putative docking site on ERK2 (p38II/ERK2, see below) nearly as well as did full-length MEK1 when each was immunoprecipitated from EGF-treated cells. Thus, the difference in activity in vitro and in transfected cells appears to be due to an impaired ability to bind to ERK2 rather than a large difference in intrinsic activity of ΔN1. This result shows that deletion of the ERK-binding domain of MEK1 not only reduces the feedback phosphorylation by ERK2, but also reduces the ability of MEK1 to phosphorylate ERK2 in vitro.

To investigate whether the ERK-binding domain is important for endogenous ERK activation, the effects of Myc-tagged full-length MEK1 and ΔN1 were tested in transiently transfected 293 cells (Fig. 5, C and D). EGF stimulated the activation of endogenous ERK1 and ERK2, measured by blotting lysates with an antibody that recognizes the phosphorylated forms of ERK1/2. Expression of full-length MEK1 in the cells had little or no effect on ERK activity in the presence or absence of EGF. However, in cells expressing ΔN1, ERK activation by EGF was significantly less than that in cells expressing MEK1 or the vector control. This inhibitory effect is probably due to sequestering of endogenous Raf-1 by ΔN1, preventing its activation of endogenous MEK. These results indicate that the ability to activate endogenous ERK proteins is impaired by the deletion of the ERK-binding domain of MEK1. Taken together, these results demonstrate that the N-terminal ERK-binding domain of MEK1 enhances not only efficient feedback phosphorylation by ERK2, but also phosphorylation and activation of ERK2 by MEK1.

We have suggested that ΔN1 phosphorylates ERK2 less well than MEK1 because it binds less well to ERK2. To obtain additional evidence in support of this conclusion, we tested the ability of wild-type MEK1 and ΔN1 to co-immunoprecipitate with ERK2 from transfected cells. The data of Fig. 6A demonstrate that whereas wild-type full-length MEK1 brings down detectable amounts of ERK2, ΔN1 does not. In agreement with the in vitro experiments of Fukuda et al. (18), these results indicate that in cells ERK2 binds to MEK1 through its N-terminal 32 residues.

Deletion of the ERK-binding Domain Does Not Reduce the Interaction of MEK1 with Endogenous Raf-1—MEK1 associates with its substrates ERK1 and ERK2 as well as its upstream activator Raf-1 in cells. The N terminus of MEK1 contains the ERK-binding domain, but the region of MEK1 that interacts with Raf-1 has not been delineated. We wanted to examine whether deletion of the ERK-binding domain of MEK1 affected its association with endogenous Raf-1. Equal amounts of Myc-tagged MEK1 and MEK1–ΔN1 were immunoprecipitated from transfected cells using an anti-Myc antibody, and the precipitates were blotted for the presence of endogenous Raf-1 (Fig. 6B). Raf-1 co-immunoprecipitated with MEK1–ΔN1 as well as it did with full-length MEK1, indicating that the N-terminal ERK-binding domain is not required for Raf-1 association. This supports the idea that ΔN1 may interfere with ERK activation by competing with endogenous MEK1 for association with Raf-1.

A MEK1-interacting Domain of ERK2 Is Localized to Its N Terminus—The N terminus of MEK1 contains the ERK-binding domain. To localize the MEK1-interacting domain of ERK2, we used three ERK2-p38 chimeras and an ERK2 deletion mutant along with full-length ERK2 as in vitro substrates for MEK1 and ΔN1 (Fig. 7). As shown above, under these conditions, ERK2 was more highly phosphorylated by wild-type full-length MEK1 than by ΔN1. However, if the MEK1-interacting domain of ERK2 was replaced by sequences from the p38 MAP kinase, the resulting chimera should be phosphorylated by both wild-type MEK1 and ΔN1 to similar extents. Based on this rationale, we compared three different ERK2-p38 chimeras and an ERK2 C-terminal deletion mutant as in vitro substrates
for immunoprecipitated MEK1 proteins (Fig. 7). p38IIERK2 contains ERK2 with its N terminus through the end of subdomain II replaced by p38; ERK2IIp38IVERK2 contains ERK2 with p38 substituted for subdomains III and IV; p38IVERK2 contains p38 substituted for ERK2 from the N terminus through subdomain IV; and ERK2–337 lacks the C-terminal 21 residues from ERK2. When equal amounts of Myc-tagged MEK1 proteins were immunoprecipitated from EGF-treated 293 cell lysates and used to phosphorylate these proteins, full-length MEK1 had 5-fold more activity than did DN1 toward wild-type ERK2, as expected. However, the difference between full-length MEK1 and DN1 in phosphorylation of the chimeras p38IIERK2 and p38IVERK2, which have the p38 N terminus, was much smaller. The chimera retaining the ERK2 N terminus, ERK2IIp38IVERK2, was phosphorylated better by full-length MEK1 than by DN1. These data suggest that a MEK1-interacting domain of ERK2 is located at its N terminus. The results with the ERK2 deletion mutant are consistent with this notion and suggest that the C terminus of ERK2 is not involved because full-length MEK1 phosphorylated ERK2 lacking the C terminus significantly better than did DN1.

**DISCUSSION**

We show here that a binding site for ERK2 at the N terminus of MEK1 enhances not only the feedback phosphorylation of MEK1-K97M phosphorylation by ERK2. A, effects of MEK1 N-terminal peptides on phosphorylation of full-length MEK1-K97M protein by active ERK2. Full-length MEK1-K97M (180 nM) was phosphorylated with 16 nM active ERK2 in the presence of the indicated concentrations of P1 or P2. Upper panel, autoradiogram; lower panel, plotted as percent maximal incorporation. One of two similar experiments is shown. B, effect of P1 on phosphorylation of full-length MEK1-K97M by active ERK2 or GST-PAK1 (232–544). Full-length MEK1-K97M (180 nM) was phosphorylated by 16 nM active ERK2 or 750 ng of PAK1 in the presence of the indicated concentrations of P1. Upper panel, autoradiogram; lower panel, plotted as percent maximal incorporation. The gel of MEK1-K97M phosphorylated by GST-PAK1 was exposed three times longer. One of three similar experiments is shown. C, effect of P1 on phosphorylation of MEK1-K97M, MBP, and Elk-1 by active ERK2. MEK1-K97M (180 nM), MBP (10 µg), and Elk-1 (immunoprecipitated with 2 µl of anti-Elk-1 antibody from 400 µg of lysates from transfected 293 cells) were phosphorylated by 16 nM active ERK2 in the presence of the indicated concentrations of P1. Phosphorylation of MBP by ERK2 had not reached saturation at 10 µg of MBP. Fractional phosphorylation of substrate in the presence compared with the absence of P1 is shown under the autoradiogram. One of two similar experiments is shown.
FIG. 5. The ERK-binding domain is required for efficient phosphorylation of ERK2 in vitro and in vivo. A, phosphorylation of 1 μg of ERK2-K52R by Myc-tagged full-length MEK1 (FL) and ΔN1 immunoprecipitated (IP) from untreated or EGF-stimulated 293 cells. Reactions were carried out at 30 °C for 30 min. B, anti-Myc antibody blot showing expression of full-length MEK1 and ΔN1. C, anti-active ERK antibody blot. The upper band is ERK1, and the lower band is ERK2. D, anti-ERK antibody blot. One of six similar experiments is shown.

MEK1 by ERK2, but also the activation of ERK2 by MEK1 in vitro and in cells. The reduced rate of phosphorylation of MEK1 lacking the binding site is not due to conformational changes that would alter phosphorylation of MEK1 by kinases other than ERK2 because PAK1, another protein kinase that phosphorylates MEK1, phosphorylates MEK1 equally well in the presence or absence of the ERK-binding site. The inhibition of EGF-stimulated ERK2 phosphorylation in cells expressing MEK1 that lacks this interacting domain indicates that the domain is also necessary for activation of ERK2 by MEK1 in intact cells. These results are consistent with a recent report examining the function of the MEK1 N terminus (26).

Multiple domains of MEK1 have now been shown to be important for activation of downstream ERK proteins in vitro. Specialized domains of MEK1 facilitate productive interactions among components of the cascade. These domains, including the MEK1 N terminus and its proline-rich insert, each appear to participate in protein-protein interactions that together serve to colocalize the cascade components in cells (9, 14). In the first identified protein kinase cascade, cAMP-dependent protein kinase activates phosphorylase kinase, which activates phosphorylase (27). Moving down the cascade, each kinase or chimeric protein is shown in parentheses above the hatched bars. The rates of phosphorylation of these proteins relative to wild-type ERK2 are as follows: p38IIERK2 (PIIE), 10%; p38IVERK2 (PIVE), 100%; ERK2–337 (EIPIVE), 30%; and ERK2–337 (337), 5–10% (see Ref. 21).

FIG. 6. The ERK-binding domain is required for co-immunoprecipitation of MEK1 with ERK2, but not with Raf-1. A, either Myc-tagged, wild-type full-length MEK1 (FL) or ΔN1 was cotransfected with hemagglutinin (HA)-tagged ERK2 in 293 cells. Proteins were immunoprecipitated (IP) with anti-Myc antibodies. Immunoprecipitates were immunoblotted with an anti-hemagglutinin antibody and, after stripping, with anti-Myc antibodies. Lysates were also blotted with an anti-hemagglutinin antibody to confirm ERK2 expression. One of two similar experiments is shown. B, Myc-tagged, wild-type full-length MEK1 and ΔN1 were immunoprecipitated from transfected 293 cells with an anti-Myc antibody, and the coprecipitating proteins were immunoblotted with an antibody to detect endogenous Raf-1. The same blot was stripped and reprobed with the anti-Myc antibody to detect MEK1 proteins. Lysates were also immunoblotted with anti-Raf-1 antibodies to confirm equal endogenous Raf-1 in the lysates. One of three similar experiments is shown.

FIG. 7. A MEK1-binding site of ERK2 is localized to its N terminus. Three ERK2-p38 chimeras and the ERK2 deletion mutant are shown schematically in the lower panel. The chimeras and wild-type ERK2 (each at 240 nM) and the deletion mutant (at 125 nM) were phosphorylated for 30 min at 30 °C by wild-type full-length MEK1 and MEK1-ΔN1 immunoprecipitated from EGF-stimulated 293 cells. Incorporation by ΔN1 is plotted as percent incorporation due to full-length MEK1; counts/min due to ERK2 and chimera autophosphorylation were subtracted from the total incorporation. Solid bars, full-length MEK1; hatched bars, MEK1-ΔN1. The number of experiments for each ERK2 or chimeric protein is shown in parentheses above the hatched bars. The rates of phosphorylation of these proteins relative to wild-type ERK2 are as follows: p38IIERK2 (PIIE), 10%; p38IVERK2 (PIVE), 100%; ERK2–337 (EIPIVE), 30%; and ERK2–337 (337), 5–10% (see Ref. 21).
for certain members of the MAP kinase family. Hallmarks of this domain are a cluster of basic residues and two leucine or isoleucine residues (see Fig. 1B, for example) (28). Therefore, we examined the effect of a MEK1-derived peptide containing this binding site on phosphorylation of other substrates by ERK2 to test the possibility that ERK2 binds to MEK1 in a manner comparable to the binding of a substrate. A peptide containing the binding site did not inhibit phosphorylation of MBP, which is not believed to contain any targeting domain, but did weakly inhibit phosphorylation of Elk-1, which contains both D and FXFP-targeting domains (25). This provides support for the idea that the ERK2-binding site of MEK1 employs a substrate-targeting domain to bind to ERK2.

Two possible explanations for the difference in inhibition seem likely. First, because Elk-1 contains an independent type of targeting sequence, interference through a D domain peptide is not sufficient to cause such great inhibition. Second, the site of binding of the D domain to ERK2 has not been unequivocally determined; two sites may exist, one within the catalytic domain and one that has been specialized for MEK recognition and that may be located at the N terminus, as suggested by our results.

Other MAP kinases also recognize targeting domains to generate high affinity interactions with substrates. Karin and co-workers (29) first identified the importance of a substrate-targeting domain by finding that different c-Jun family members were differentially phosphorylated by the c-Jun N-terminal kinase/stress-activated protein kinase. The difference was due to the presence or absence of a targeting domain in the substrate, just N-terminal to its phosphorylation sites, that bound to the kinase. They subsequently showed that this region of c-Jun bound with higher affinity to a particular splice variant of the Jun N-terminal kinase/stress-activated protein kinase that contained an insert between subdomains IX and X in the C-terminal portion of the kinase catalytic core (30).

Based on three-dimensional structures of protein kinases (31, 32), this part of the catalytic domain is involved in binding protein substrates. Therefore, these findings suggested that the targeting domain of a substrate recognized a particular sequence within the kinase catalytic core, the normal function of which was to recognize protein substrates. Our results with ERK2-p38 chimeras and a deletion mutant indicate that a D domain-like sequence of MEK1 binds to ERK2 at least in part through an N-terminal sequence of ERK2. Our findings are consistent with those of Bardwell et al. (33, 34), who reported that yeast MAP kinases interact with the yeast MEK Ste7p through residues outside its catalytic domain. Thus, it may be possible that substrate-interacting domains bind to their cognate protein kinases in regions outside the kinase active site or catalytic core. Support for this idea comes from the fact that substrate-targeting domains like the D domain may have any number of orientations relative to the sites that are phosphorylated by the kinase (25, 28). Reports on protein kinases such as myosin light chain kinase suggest that sequences outside the catalytic core are also important for recognition of their substrates (35). Thus, future experiments will explore the possibility that MAP kinases may bind substrates through accessory sequences outside the catalytic domain.

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