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Mitogen-activated protein kinase (MAPK)-docking sites in MAPK kinases function as tethers that are crucial for MAPK regulation in vivo

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Abstract

Docking sites on targets of mitogen-activated protein kinases (MAPKs) facilitate accurate and efficient substrate phosphorylation. MAPK/ERK kinases (MEKs, or MKKs), the upstream regulators of MAPKs, also contain N-terminal MAPK-docking sites, or 'D-sites'; however, the in vivo functions of MEK D-sites are incompletely understood. Here we found that the ability of constitutively-active human MEK1 and MEK2 to stimulate ERK phosphorylation and to induce the neoplastic transformation of NIH 3T3 cells required the integrity of the D-site. In addition, D-site mutants of otherwise wild-type MEK1/2 were unable to anchor unphosphorylated ERK2 in the cytoplasm. ERK activation, cytoplasmic anchoring and release were completely retained in 'swap' mutants in which MEK2's D-site was replaced with the D-site of MEK1 or yeast Ste7. Furthermore, these abilities were significantly retained when MEK2's D-site was moved to its C-terminus, or replaced by an unrelated MAPK-binding domain taken from the Ets-1 transcription factor. We conclude that the D-sites in MEKs are crucial for the activation of their cognate MAPKs in vivo, and that their primary function is to tether their cognate MAPKs near the MEK's kinase domain. This proximity effect is sufficient to explain the contribution that the D-site interaction makes to several biologically important signaling events.

Keywords

Mitogen-activated protein kinases; Signal transduction; Phosphorylation; Binding sites; Protein binding

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades are found at the heart of numerous networks that control cellular processes such as proliferation, differentiation, cell survival and cell death [1–3]. The basic core of each cascade includes a MAPK kinase kinase (MAPKKK, MAP3K, or MEKK) that phosphorylates and activates a MAPK/ERK kinase (MAPKK, MAP2K, MKK, or MEK), which in turn activates a MAPK. The activated MAPKs then phosphorylate transcription factors, protein kinases and other proteins, leading to changes in cell physiology and gene expression. The wide variety of stimuli that elicit MAPK activation, together with the huge number of distinct responses that MAPKs regulate, raise the issue of how MAPKs interact with their upstream and downstream partners to achieve specific coupling of signal to the appropriate cellular response [4].

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One of the most studied mammalian MAPK pathways is the ERK1/ERK2 pathway [5]. This pathway can be activated by a variety of extracellular signals, and plays a crucial role in numerous biological processes, notably those involving growth and differentiation. Stimulation of this pathway is typically initiated by receptor-mediated activation of the small G-protein Ras, which binds to and recruits the serine/threonine kinase Raf to the plasma membrane, where Raf becomes fully activated. Raf then phosphorylates and activates MEK1 and MEK2. Activated MEK1 and MEK2, in turn, phosphorylate, and thereby activate, ERK1 and ERK2 on a tyrosine and threonine residue located in their activation loop. Unlike Raf and MEK1/2, which remain largely cytoplasmic, ERK1/2 translocate into the nucleus following activation [6]. The Raf/ERK cascade is activated in many human cancers [7,8]. Furthermore, MAPK cascades have been found to be dysregulated in a number of pathological conditions. Accordingly, MAPK cascades provide promising possibilities for the development of therapies for cancer and other diseases [9–12].

Three other fully-elaborated, distinct MAPK cascades have been identified in mammals. These include the p38 pathway, which can be activated by MKK3, MKK6 and MKK4, the JNK pathway which is activated by MKK4 and MKK7, and the ERK5 pathway that is activated by MEK5 [13–16]. Generally, the MEKs in one cascade do not phosphorylate the MAPKs in other cascades. How this selectivity is achieved is incompletely understood.

Signaling through individual MAPK cascades is promoted by a series of protein–protein interactions that lead to specific activation of the correct kinase. The most important recognition event is that between the active site of the kinase and the target phosphoacceptor residue and nearby residues in the substrate [17]. However, other molecular determinants appear to be involved in directing individual kinases towards the correct substrate. MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 have been shown to bind to their target MAPKs with low-micromolar affinity ($K_d \sim 5\text{--}50\ \mu\text{M}$ depending on the particular pair) [18–20]. MEK–MAPK complex formation is attributable both to the interaction of the kinase domain of the MEK with its cognate MAPK, and to a distinct docking interaction involving the N-terminal, non-catalytic domain of the MEK [21–25]. The N-terminus of most MEKs contains a short conserved sequence (consensus $[\text{K/R}]_2\text{--}3\text{--X}_{1\text{--}6}\text{--}[\text{L/I}]\text{--X}\text{--}[\text{L/I}]$), designated the ‘MAPK-docking site’ or ‘D-site’, that was first recognized in the yeast MEK Ste7 [26]. The D-site in Ste7 is necessary and sufficient for high-affinity binding to its cognate MAPKs Fus3 and Kss1 [20,21]. Similarly, the D-sites in mammalian MEK1, MEK2, MKK3, MKK4 and MKK6 are necessary and sufficient for high-affinity binding to their cognate MAPKs [18,20,27,28]. Residues in the D-site make contacts with regions of the MAPK that are relatively distant from the MAPK activation loop [29].

Several studies, employing mainly in vitro biochemical approaches, have found that interfering with MEK–MAPK docking reduces the efficiency of MEK-mediated MAPK phosphorylation [18,20,27,28,30–32]. However, many unanswered questions remain about the function of MEK–MAPK docking in vivo in mammalian cells. For instance, does MEK–MAPK docking function primarily to enhance signal transmission from the MEK to the MAPK, the specificity of MEK → MAPK signaling, or both? Alternatively, might MEK–MAPK docking have an altogether different function, such as promoting the regulated localization of the MAPK [31,33], or the feedback phosphorylation of the MEK [27]? Furthermore, is the main function of the D-sites in MEKs to bind to their cognate MAPKs, or do they serve some structural role in the activity or integrity of the kinase domain of the MEK [34–37]? Finally, is MEK–MAPK docking, in itself, important for efficient signal transmission in vivo, or is its role largely redundant with that of scaffold proteins that bind to both the MEK and the MAPK [20,38]?

It is also uncertain how MEK D-sites work, mechanistically. Do D-sites ensnare the MAPK and hold it in close proximity to the kinase domain of the MEK, or is it important that they display the MAPK in a particular quaternary structure and stereochemical orientation? In other words, does a MEK D-site present its cognate MAPK to the MEK's kinase domain in an optimal conformation and precise configuration, or does it simply tether the MAPK near the MEK's kinase domain in a high local concentration? MAPK-docking sites on transcription factors such as Elk-1 and c-Jun have been shown to be flexible, modular entities that can be moved or exchanged [39,40], consistent with the idea of tethering. But it is not clear that MEK D-sites should necessarily function similarly, given (1) the extremely high specificity of MEK–MAPK transactions [41–43]; (2) the complicated series of events that may be orchestrated by MEK D-sites, including the regulated subcellular anchoring and release of their cognate MAPKs [22,31,33,44–47]; (3) the suggestions that MEK D-sites may play a structural role in MEK integrity [34–37]. Furthermore, unlike D-sites in substrates, which are found at variable positions throughout the protein [48], the D-sites in MEKs are uniformly located at or near the amino terminus [20,26]. This suggests the possibility of significant positional constraints to MEK D-site function, inconsistent with a simple tethering role.

Here we report a series of experiments that examine MEK D-site function in vivo and provide significant insight into some of these questions.

2. Materials and methods

2.1. Materials and antibodies

Tissue culture media, trypsin and antibiotics were purchased from Gibco BRL. Mouse monoclonal anti-V5 was purchased from Invitrogen and goat polyclonal anti-ERK1/2 and anti-phosphospecific ERK1/2 were obtained from Cell Signaling. AlexaFluor 488- and 594-conjugated antibodies were from Molecular Probes Inc. and HRP-conjugated secondary antibodies were from Pierce. All other standard reagents and chemicals were from Sigma or Fisher.

2.2. Plasmids

To construct full-length pcDNA3.1-V5-MEK1 and pcDNA3.1-V5-MEK2, the appropriate coding sequences were amplified by high-fidelity PCR using either pBS-MEK1 or pBS-MEK2 [49] as template and primers LB111 and MEK1a or LB114 and MEK2a, respectively (see Table 1 for primers). The resulting products were digested with either *Bam*HI and *Xho*I or *Eco*RI and *Xho*I, for MEK1 and MEK2 respectively, and were then ligated into the corresponding sites of pcDNA3.1-V5 (Invitrogen). Docking-site deficient versions of the above constructs were obtained by amplifying the appropriate sequences from either pBS-MEK1 or pBS-MEK2 using primers LB112 and MEK1a or LB115 and MEK2a, respectively. The resultant products were ligated as above into pcDNA3.1-V5. For docking site swaps of MEK1/2, the required nucleotide residues were introduced into the 5' ends of MEK1 or MEK2 using the extended primers, MEK1–MEK2ds, MEK2–MEK1ds or MEK2–Ste7ds and the appropriate downstream primer. The resultant fragments were ligated as above into pcDNA3.1-V5. To obtain constitutively active MEK1 and MEK2, oligonucleotide-directed mutagenesis was performed in order to introduce mutations that corresponded to Ser218Glu and Ser222Asp for MEK1, and Ser222Asp and Ser226Asp for MEK2. Full-length MKK3 (pcDNA3.1-V5-MKK3) was obtained from Invitrogen. To generate docking site deficient MKK3, primers MKK3del and MKK3b were used to amplify the appropriate coding sequence from pcDNA3.1-V5-MKK3 and the resultant fragment was ligated into the *Eco*RI and *Xba*I sites of pcDNA3.1-V5. Full-length MKK6 cDNA was obtained from the I.M.A.G.E. Consortium. Full-length and docking site deficient MKK6

sequences were amplified using primers MKK6a and MKK6b, or MKK6del and MKK6b respectively and the fragments were cloned into the *KpnI* and *NheI* sites of pcDNA3.1-FLAG (a gift from R. Sainson, University of California, Irvine). Constitutively active versions of MKK3 and MKK6 were generated using oligonucleotide-directed mutagenesis. MEK2^{EEAA} in which the critical MAPK binding residues had been mutated (R4E, R5E, L12A and I14A) was generated by site-directed mutagenesis. MEK2-DDΔ-C-dock was constructed by ligating the MEK2-CDOCK(1)/MEK2-CDOCK(2) oligo adapter into the *AgeI/PmeI* sites of pcDNA3.1-V5-MEK2-DDΔ. The MEK2-Ala construct, which contained alanine residues in place of all docking site residues, was also created by oligo adapter cloning, using *EcoRI/AatII* oligos MEK2-Ala(1)/MEK2-Ala(2). These adapters were then ligated with *AatII/XhoI* digested MEK2-DDΔ (amplified from pBS-MEK2 using primers MEK2-link and MEK2a) and *EcoRI/XhoI* digested pcDNA3.1-V5. The pcDNA3.1-V5-MEK2-DDΔ-Ets1 plasmid was constructed by amplifying the Pointed domain sequence from pOTB7-Ets1 (Open Biosystems) using primers Ets1a and Ets1b. This fragment was then digested with *HindIII* and *AatII* and ligated with MEK2-DDΔ (amplified and digested as above) and with *HindIII/XhoI* digested pcDNA3.1-V5. Full length FLAG-tagged ERK2 was constructed by amplifying coding sequence for ERK2 from pBS-ERK2 [21] using primers ERK2a and ERK2b and ligating the resulting product into the *KpnI* and *NheI* sites of pcDNA3.1-FLAG. The authenticity of all plasmids was confirmed by DNA sequencing.

2.3. Cell culture and transfection

HEK293, NIH3T3 and HeLa cells were obtained from ATCC and were cultured at 37 °C in a humid atmosphere containing 5% CO₂ in air. Cells were grown in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturers guidelines.

2.4. Transformation of NIH3T3 cells

Cells (3×10⁶) were seeded into 10 cm² dishes and grown overnight. Transfections were performed with 6 µg of the appropriate plasmid construct using Lipofectamine (according to the manufacturer's instructions). Cells were grown for 21 days, changing media every 3 days. Following this period, the media was removed and the cells were washed with PBS and fixed in acetone:methanol (1:1) for 5 min. The cells were then stained with methylene blue (0.1% w/v) for 3 min. The transformed foci, which appeared as dark blue colonies, were counted and expressed as number of colonies per 10 cm² dish.

2.5. SDS-PAGE and Western blotting

Proteins (20 µg per well) were separated on standard SDS–polyacrylamide gels. For Western blot analysis, proteins were transferred to PVDF membranes, then blocked in 1% BSA in TBS–0.1% Tween for 1 h. Primary antibody incubations were carried out overnight at room temperature, followed by 1 h incubations with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using an ECL detection kit (Pierce) according to the manufacturer's instructions. For quantification of ERK phosphorylation levels, AlexaFluor-680/800 nm secondary conjugates were used and PVDF membranes were analyzed using the Odyssey Infra-Red Imaging System and software (Li-Cor BioSciences) according to the manufacturer's instructions.

2.6. Immunofluorescence microscopy

The method for immunofluorescence microscopy was adapted from previous reported methods [50]. Cells were grown on glass coverslips overnight. Media was removed and the cells were washed with pre-warmed (to 37 °C) PBS and fixed in pre-warmed 10% formalin

in neutral buffered saline (approximately 4% formaldehyde, Sigma) for 5 min. All subsequent steps were performed at room temperature. After fixation, the cells were permeabilized with 0.1% Triton-X100 in PBS for 5 min and fixed once again for 5 min. The cells were then washed three times with PBS and incubated in sodium borohydride solution (1 mg/ml in PBS) for 5 min to reduce autofluorescence. Following three further PBS wash steps, the cells were blocked in 5% rabbit serum in PBS for 3 h. The cells were then incubated with primary antibody (diluted into PBS–5% serum) overnight followed by AlexaFluor 488- or 564-conjugated secondary antibody for 3 h, with eight PBS washes performed in between incubations. The cells were then washed eight times with PBS and mounted onto slides in Vectashield mounting medium. Cells were viewed using a Zeiss Axiovert 200 M fluorescence microscope and images were captured using a Photometrics CoolSnap HQ digital camera (Roper Scientific). Images were processed with the Metamorph image analysis program (Universal Imaging Corp.).

2.7. In vitro transcription, translation and in vitro binding assays

MEK peptides labeled with [³⁵S]-methionine were produced by a coupled in vitro transcription and translation system (STP3 T7, Novagen). Translation products were partially purified by ammonium sulfate precipitation and quantified as described [20,21]. Production of GST fusion proteins and GST co-sedimentation assays were performed as described previously [20]. Results were analyzed by SDS-PAGE and quantified using a PhosphorImager to determine the amount of each translation product bound as a percentage of input.

3. Results

3.1. Deletion of docking sites from constitutively-active MEK proteins inhibits their ability to phosphorylate their endogenous target MAPKs

Some evidence regarding the importance of MEK D-sites for MAPK phosphorylation in vivo has been provided by others [27,28]. Xu et al. showed that overexpression of a MEK1 derivative lacking its first 32 residues inhibited ERK phosphorylation, presumably by acting as a dominant negative [27]. Furthermore, Enslen et al. observed reduced kinase activity of p38 immunoprecipitated from cells following co-transfection with D-site mutants of MKK3 or MKK6 [28]. MEK D-sites have also been shown to be important in MEK-mediated MAPK phosphorylation in several studies that used in vitro protein kinase assays [18,20,30–32]. However, in some cases the observed effects have been rather modest; for instance, Tanoue et al. observed only a 30% decrease in the activity of MEK1 with a mutant D-site [32]. Furthermore, mutation of the D-site in yeast Ste7^{MEK} resulted in only a very modest in vivo phenotype, unless the Ste5 scaffold protein was also compromised [20].

Hence, to clarify the importance of MEK D-sites in MAPK activation in vivo in mammalian cells, the following approach was taken: constitutively-active, full-length MEK proteins were expressed in human epithelial cells (HEK293) and their ability to activate their endogenous MAPK targets was compared to that of otherwise identical MEK proteins that were deleted of their D-sites; Fig. 1A shows the D-sites of the relevant MEKs. The introduced MEK proteins were expressed at levels roughly comparable to that of the corresponding endogenous MEK proteins (data not shown). The phosphorylation of the relevant MAPK proteins was monitored by phosphorylation-state specific antibodies that specifically recognize the dually-phosphorylated MAPKs [51]. MEKs are activated by phosphorylation of serine and threonine residues in their activation loop by upstream MEK kinases such as Raf [52,53]. Potent, constitutively-active versions of MEK1, MEK2, MKK3 and MKK6 can be created by the mutation of these residues to aspartate or glutamate, thereby mimicking the negative charge entailed by phosphorylation [34,36,54,55]. As shown

in Fig. 1B, when expressed in serum-starved cells, MEK1-ED, which contains an S218E S222D double mutation, activated endogenous ERK1 and ERK2. Notably, expression of MEK2-DD (S222D S226D) caused a high degree of ERK1/2 phosphorylation, comparable to the maximal amount of phosphorylation seen following treatment with serum for 15 min. In contrast, constitutively active MEK1 and MEK2 that lacked docking sites (MEK1-ED Δ_{3-11} and MEK2-DD Δ_{4-14}) showed a very little ability to activate ERK2 (Fig. 1B), and showed no detectable activation of ERK1. The decrease in ERK activation caused by the D-site deletion was greater than nine-fold, as determined by quantification of immunoblots using fluorescent antibodies (see Materials and methods for details).

To confirm the importance of the D-sites in MKK3 and MKK6 for the phosphorylation of the p38 MAPK, constitutively-active MKK3 (MKK3-EE; S218E T222E) and MKK6 (MKK6-EE; S227E T231E) were expressed in human cells. These MEKs stimulated the activation of endogenous p38, as determined by immunoblotting with an antibody specific for dually-phosphorylated p38 (Fig. 1C). In contrast, D-site-deleted mutants of constitutively active MKK3 (MKK3-EE Δ_{1-27}) and MKK6 (MKK6-EE Δ_{1-15}) did not stimulate p38 activation (Fig. 1C). These results on the phosphorylation of endogenous p38 are consistent with those of Enslen et al. [28], who examined the kinase activity of overexpressed p38.

To summarize, in both the ERK1/2 and p38 MAPK pathways, MEK D-sites are critical for efficient signaling from constitutively-active MEK proteins to their cognate MAPKs in vivo.

3.2. D-site mutants are unable to cause morphological transformation or induce focus formation in NIH3T3 cells

To determine requirement of the MAPK-docking site for a complex cellular endpoint, we examined the ability of D-site-deleted MEK1 and MEK2 proteins to potentiate neoplastic transformation. Previous work has shown that, similar to activated Ras, constitutively active MEK1 and MEK2 are able to cause accelerated growth and transduction of mitogenic signals, resulting in neoplastic transformation and focus formation [34,36,56]. In confirmation of these prior results, we observed that both constitutively-active MEK1 and constitutively-active MEK2 proteins caused morphological transformation of NIH3T3 cells (Fig. 2A, panels i and iv). In particular, constitutively active MEK2 caused a high degree of morphological transformation, comparable to that caused by activated H-Ras (Fig. 2A, panel vi). In contrast, cells transfected with the docking site-deleted versions showed no obvious cellular transformation (Fig. 2A, panels ii and v). Quantification of the degree of transformation, as measured by focus formation (Fig. 2B and C), revealed that MEK2-DD expression resulted in approximately 50% more foci than MEK1-ED expression (37 ± 6 compared to 24 ± 4). Deletion of the docking site from MEK1 or MEK2 resulted in an 82% and 80% reduction in foci formation, respectively (from 24 ± 4 to 4 ± 2 , and from 37 ± 6 to 8 ± 3). Thus, MEK D-sites play a strongly positive role in neoplastic transformation caused by constitutively-active MEK proteins.

3.3. Cytosolic retention of ERK2 is dependent on the docking sites within MEK1 and MEK2

In mammalian cells, both MEK1/2 and ERK1/2 are present at comparable levels of about 500,000–1,000,000 molecules/cell [57]. In the absence of mitogenic signals, ERK is predominantly cytosolic, and is believed to be retained in the cytoplasm by its direct interaction with MEK [22,46]. When ERK is overexpressed, it accumulates in the nucleus, presumably because it is now in stoichiometric excess of the MEK [22,33,58]. Expression of additional wild-type MEK1 was shown to reverse this effect, sequestering ERK2 in the cytoplasm, whereas MEK1 derivatives with mutated D-sites were unable to restrict ERK to the cytoplasm [31]. Hence, this experimental approach provides a visual read out of D-site-dependent MEK–ERK binding in vivo.

As shown in Fig. 3, when grown in serum-containing media, over-expressed ERK2 was distributed equally between the cytosol and the nucleus. Co-expression of either full length MEK1 or MEK2 promoted the cytosolic retention of ERK2 (Fig. 3B and D), resulting in very little nuclear ERK2. Wild-type MEK1 and MEK2 proteins that lacked their docking sites however, were unable to mediate the cytosolic retention of ERK2 (Fig. 3C and E), consistent with their demonstrated defect in ERK binding in vitro [20]. These data confirm previous findings with MEK1 [31], and extend them to MEK2.

3.4. MEK-mediated ERK activation can be restored by the addition of alternative docking sites from other MAP kinase kinases

Do MEK D-sites function modularly? That is, can they be exchanged with D-sites in other MEK proteins, moved to other parts of the protein, or replaced with other ERK-binding domains thought to perform similar functions? To begin to address these questions, we first asked if the core D-sites could be swapped between different MEKs (Fig. 4A, B). For instance, could MEK1's D-site be replaced with the D-site of MEK2, and visa-versa? An initial swap of MEK2 and MEK1 docking sites onto constitutively active MEK1 and MEK2, respectively, demonstrated that both proteins were able to function and activate ERK1/2 as well as the wild-type proteins (Fig. 4B). In fact, fusion of the MEK2 docking site onto MEK1-ED Δ resulted in a protein that was a better activator of ERK1/2 than full-length MEK1-ED, and fusion of the MEK1 docking site onto MEK2-DD Δ resulted in a protein that was a slightly worse activator of ERK1/2 than full-length MEK2-DD (Fig. 4B). These results correlate the with higher affinity of the MEK2 D-site for ERK2, as measured in peptide competition assays [30], and suggest that binding affinity may roughly correlate with signal strength.

The yeast MEK Ste7 is a presumed ortholog of MEK1 and MEK2 [59]. Indeed, the D-site of Ste7 binds with high affinity to mammalian ERK2 in vitro [20]. Fusion of either the first 19 residues of Ste7, or the core of Ste7's D-site (residues 9–17), to constitutively-active MEK2 in place of MEK2's own D-site, also resulted in a protein that was able to activate endogenous ERK1/2 to essentially wild-type levels (Fig. 4D and data not shown).

Hence, the substantial defect in MAPK activation that resulted from deleting the D-site from MEK1 or MEK2 could be suppressed by replacing the deleted region with the D-site from another MEK. A control protein, which contained a stretch of 11 alanine residues in place of the MEK2 docking site (MEK2-Ala) was not able to mediate any ERK activation (Fig. 4D). In addition, another control protein, MEK2^{EEAA}, which contains mutations (R4E, R5E, L12A and I14A) in the critical MAPK binding residues, showed little ability to activate ERK1/2 (Fig. 4D). Hence the observed suppression was not due to a restoration of the spacing between the very N-terminal residues and the rest of the protein, nor to a restoration of the α -carbon backbone. Most likely, the observed suppression was due to a restoration of D-site function.

3.5. The effects of docking site location within the protein

Next we asked if the N-terminal D-site of MEK2 would function effectively when placed at the C-terminus of the protein instead. The MEK2 D-site was fused to the C-terminus of constitutively-active MEK2 that was lacking its N-terminal D-site, creating MEK2-DD Δ /C-dock (Fig. 5A). This manipulation restored the ability of the protein to phosphorylate endogenous ERK1 and ERK2 (Fig. 5B), although not completely. ERK1 and ERK were phosphorylated by MEK2-DD Δ /C-dock to $40 \pm 7\%$ of the level obtained with 'wild-type' MEK2-DD protein. This result indicates that the N-terminal location of the docking site is not critical for its function.

3.6. The ERK-binding domain from the transcription factor Ets-1 can also mediate MEK-dependent ERK activation

As a final means to explore the potential flexibility of MEK–MAPK docking, we asked if a distinct type of ERK-binding domain could functionally replace the MEK D-site. The Ets-1 transcription factor, a member of the ETS family of evolutionarily-related proteins, has been shown to contain an ERK2-docking site within its 80-amino acid Pointed (PNT) domain [60]. Unlike the consensus D-site found in MEKs, this docking site contains a LXLXXXXF motif, of which the F is the most critical residue. Moreover, these residues lie on the surface of the globular PNT domain.

We tested if the PNT domain of Ets-1 could mediate ERK binding/activation and thereby restore the activity of D-site-deleted MEK2. Residues 54–138 of Ets-1, encoding the entire PNT domain of Ets-1, were fused to the N-terminus of constitutively active MEK2 that lacked its own D-site (Fig. 6). Expression of this fusion protein (Ets-1-MEK2-DDA) indicated that it was indeed able to potentiate the activation of ERK1/2 in the absence of serum (Fig. 6B), although at a level that was somewhat lower than the ‘wild-type’ constitutively active MEK2 (59±14% of full-length). The Ets-1-MEK2-DD fusion protein was also able to drive the nuclear accumulation of endogenous ERK2 (Fig. 6D), similar to the full-length constitutively active MEK2. This nuclear translocation of endogenous ERK mimicked the translocation event seen in response to the addition of serum (Fig. 6D, control +). In contrast, constitutively active MEK2 that lacked its docking site was unable to mediate this nuclear translocation process. Furthermore, an Ets-1-MEK2 fusion that was not constitutively active was able to mediate the cytosolic retention of ERK2 (Fig. 6E), similar to wild-type MEK2 protein (Fig. 3D), but unlike D-site-deleted MEK2 (Fig. 3E). These results indicate that the Ets-1 PNT domain, when fused to MEK2, can bind directly to ERK2, and can replace the function of the MEK2 docking site for signal transmission, cytosolic anchoring, and release.

3.7. Partial restoration of ERK binding in the reconfigured MEK proteins

To determine the affinity with which the redesigned MEK proteins were binding to ERK, GST co-sedimentation assays were performed (Fig. 7). Purified recombinant GST-ERK2 (or GST as a control) bound to glutathione–Sephadex beads was incubated with either full-length constitutively active MEK2 or the indicated MEK2 variants. Bead-bound complexes were collected by sedimentation and analyzed by SDS-PAGE and autoradiography. As demonstrated previously [20] full-length MEK2 bound effectively ($K_d \sim 5 \mu\text{M}$) to GST-ERK2, but not to GST (Fig. 7A). In contrast, docking-deficient MEK2 bound only weakly to GST-ERK2 (17±7% of full-length MEK2 binding). Restoration of binding to approximately 36±9% could be achieved by fusion of the Ets1 ERK-binding domain to the N-terminus of docking site deficient MEK2. Similarly, fusion of a C-terminal MEK2 docking site to docking site-deficient MEK2 was able to modestly restore binding to GST-ERK2, confirming the in vivo activity seen previously for this protein (Fig. 5). A control MEK2^{EEAA} protein, in which the critical MAPK binding residues had been mutated (R4E, R5E, L12A and I14A), showed little binding to GST-ERK2, demonstrating the requirement for specific interactions mediated by the key conserved residues of the D-site (Fig. 7). It was notable that neither of the redesigned MEK variants (MEK2-C-dock and Ets-MEK2) was able to bind to ERK2 with the same affinity as wild type MEK2 (Fig. 7). This correlated with their partial ability to restore function in quantitative ERK phosphorylation assays (Figs. 5 and 6). In summary, the above results demonstrate that an alternative MAPK-binding domain can functionally replace a MEK D-site, suggesting that MEK D-sites are modular, flexible components that act to tether MEKs in close proximity to their cognate MAPKs.

4. Discussion

MAPK pathways play pivotal roles in regulating a diversity of cellular functions. Recent studies suggest that specific protein interactions between MAPKs and their activators, substrates, scaffold proteins and deactivators are achieved through high-fidelity docking sites present on MAPK-interacting proteins [48,61]. MEK proteins, the direct activators of MAP kinases, contain N-terminal MAPK-docking sites (D-sites) that are involved in several aspects of MAPK regulation. Here we tested a proximity, or tethering, model of MEK D-site function.

4.1. MEK D-sites are crucial for MAPK regulation

First, to lay the groundwork for subsequent experiments, we verified the importance of MEK D-sites in MEK→MAPK transactions *in vivo*. Using constitutively-active MEK proteins containing wild-type or mutant D-sites, we found that deletion of the D-sites from MEK1, MEK2, MKK3 and MKK6 dramatically compromised their ability to phosphorylate their cognate MAPKs in mammalian cells in tissue culture (Fig. 1). Furthermore, removal of the D-site from mutationally-activated MEK1 or MEK2 reduced their ability to neoplastically transform NIH-3T3 cells (Fig. 2). In addition, D-site-deleted MEK1 and MEK2 were unable to act as cytoplasmic anchors and retain unactivated ERK1/2 in the cytoplasm (Fig. 3). Collectively with previous studies [22,27,28,31], these results indicate that MEK D-sites are essential for mediating MEK–MAPK transactions *in vivo*.

4.2. MEK D-sites function as tethers for MAP kinases

We then asked whether a tethering mechanism could account for the *in vivo* functions of MEK D-sites. We found that MEK D-sites are indeed modular components that act to tether the kinase domain of the MEK in close proximity to its cognate MAPK. The modularity and flexibility of MEK D-sites was demonstrated in three ways. First, when the native D-sites of MEK1 or MEK2 were replaced with heterologous D-sites, the resulting chimeric MEKs retained full function (Fig. 4). Second, fusion of the MEK2 docking site to the C-terminus of MEK2 restored the ability of MEK2 to activate ERK1/2, suggesting that docking site location is not crucial for its function (Fig. 5). Finally, the native ‘D-type’ docking site of MEK2 was replaced by the ERK-binding domain from the Ets-1 transcription factor. This docking domain, which contains an LXLXXXF motif, differs considerably from other MAPK docking sites [60]. Nonetheless, this docking domain restored several biological activities to docking-site deficient MEK2, including the ability to anchor unphosphorylated ERK in the cytoplasm, the ability to efficiently phosphorylate ERK upon serum stimulation, and the ability to release this active ERK2 from cytoplasmic anchoring so that a portion of it could translocate to the nucleus (Fig. 6).

Because MEK–MAPK transactions were partially tolerant to presumably substantial perturbations of the architecture and stereochemistry of the complex, and because a heterologous docking interaction was able to functionally replace the native one, we conclude that a simple tethering mechanism is largely sufficient to account for multiple aspects of MEK D-site function. The lack of constraints on the position, orientation and exact sequence of the D-site for multiple aspects of MEK D-site function is remarkable and unanticipated, since, even in substrates, there are constraints on the position of docking sites relative to the target phosphoacceptor residues [40].

4.3. Docking and scaffolding

Previously, we studied the *in vivo* function of MEK D-sites in yeast mutants containing Ste7^{MEK} with a defective D-site [20]. This study demonstrated that compromising the Ste7 D-site, by itself, had little or no effect on the yeast pheromone response. Only when

docking-defective Ste7 mutants were combined with mutants of the Ste5 scaffold protein was a significant signaling defect observed. Hence, in this system, MEK–MAPK docking, and the binding of both MEK and MAPK to a scaffold protein, play mutually-reinforcing roles. In contrast, in the present study, D-site function was strongly required for MEK→MAPK signal transmission, even in the presence of endogenous scaffold proteins that can bind to both MEK and ERK, such as MP1, KSR and MORG [62–65]; and p38 scaffolds such as JIP2, JLP and OSM [66–68]. These results suggest that scaffolding cannot substitute for MEK–MAPK docking in mammalian cells. A caveat to this conclusion is that it is based on experiments that used constitutively-active MEK proteins; perhaps some scaffolds can substitute for MEK D-site function in transient, but not sustained, MEK→MAPK signaling.

4.4. Are MEK D-sites just MAPK tethers?

Contrasting the view that MEK D-sites simply serve as flexible tethers for MAPKs is the view that they make intimate and important contacts with the MEK's kinase domain. The 3-D structures of MEK1 and MEK2 have recently been published [69], but these structures lack the N-terminal domains that contain the D-sites. Conceivably, the MEK N-terminus could make direct stabilizing contacts with the kinase domain, as has been observed for other kinases [70,71]. Indeed, this idea has been proposed for MEK1 and MEK2 [34–37]. Our results indicate that such contacts, if they exist, certainly do not depend on the precise amino acid sequence of the D-site of a particular MEK, because we observed that full activity was maintained when the D-sites were swapped between MEK1, MEK2 and yeast Ste7. Indeed, MEK1 activity increased when its D-site was replaced by the D-site of MEK2 (Fig. 4). Furthermore, replacement of the 14-residue MEK2 D-site with the unrelated 85-residue PNT domain from Ets-1 did not substantially disturb MEK2's catalytic function, suggesting that D-site residues do not make key functional contacts with the kinase domain of the MEK.

On the other hand, it can be argued that when the MEK2 D-site was moved to its C-terminus, or replaced with the Ets-1 PNT domain, MEK2 function was not fully restored (40% and 60% respectively). Perhaps what was *not* restored was a key interaction of the D-site with the MEK kinase domain, or the precise stereochemistry of the MEK–MAPK complex. Although this may be true, it should be noted that both the C-terminal docking site fusion and the Ets-1 fusion only partially restored MAPK binding to MEK2 (Fig. 7). Hence, the observations remain consistent with the idea MEK D-sites are flexible tethers that simply function to bind MAPKs, and that a certain affinity of binding is required for full function.

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Fig. 1. Deletion of the docking site from active MEK proteins inhibits their ability to phosphorylate their target MAPKs. (A) Sequence of MEK1, MEK2, MKK3 and MKK6 N-termini, with the core of their MAPK-docking sites highlighted in grey. The most N-terminal residue shown is indicated on the left. (B) HEK293 cells were transfected with plasmids encoding full-length constitutively active MEK1 or MEK2 (MEK1-ED and MEK2-DD, respectively), docking site deficient constitutively active MEK1 or MEK2 (MEK1-EDΔ and MEK2-DDΔ, respectively) or control empty vector (vector control). Twenty-four hours post-transfection cells were transferred to low-serum (0.5%) medium and grown for a further 24 h. Cells were then harvested and lysed and ERK1/2 phosphorylation was analyzed by Western blotting. For serum activation, cells were incubated with complete medium (10% serum) for 15 min prior to harvesting. (C) Cells were transfected with plasmids encoding full-length constitutively active MKK3 or MKK6 (MKK3-EE and MKK6-EE), docking site deficient active MKK3 or MKK6 (MKK3-EEΔ and MKK6-EEΔ) or control empty vector (vector control). Cells were lysed 48 h post-transfection, and p38 phosphorylation was assessed by Western blotting. For stimulation, cells were incubated for 30 min with 100 ng/ml anisomycin.

Fig. 2.

Docking-site mutants of MEK1 and MEK2 are unable to induce the morphological transformation of NIH3T3 cells. NIH3T3 cells were transfected with plasmids encoding full-length constitutively active MEK1 or MEK2 (MEK1-ED and MEK2-DD, respectively), docking site deficient constitutively active MEK1 or MEK2 (MEK1-ED Δ and MEK2-DD Δ , respectively), activated V12-Ras (H-Ras V12) or empty vector (control). (A) Twenty-one days post-transfection cells were viewed using an IX70 Olympus light microscope and morphological transformation was assessed. (B) Cells were fixed and permeabilized and then stained with 0.1% methylene blue to visualize foci. (C) Quantification of the number of foci formed per 10 cm dish ($\pm$ standard deviation, $n = 3$).

Fig. 3.

The MEK-mediated cytosolic retention of ERK2 is dependent on docking sites. HeLa cells were grown on coverslips and were co-transfected with plasmids encoding ERK2 and either full-length or docking-site deficient MEK1 or MEK2 (V5-tagged). Forty-eight hours post-transfection cells were fixed and permeabilised and incubated with monoclonal anti-V5 and rabbit polyclonal anti-ERK1/2 antibodies followed by anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 564 secondary antibodies. Scale bar=10 μ m.

Fig. 4.

Replacement of MEK1/2 docking sites with other MKK docking sites can restore ERK activation. (A) Alignment of N-terminal sequences of MEK2, MEK1 and the yeast MEK protein, Ste7, with conserved docking site regions highlighted in grey. The most N-terminal residue shown is indicated on the left. (B) HEK293 cells were transfected with full-length constitutively active (V5-tagged) MEK1 or MEK2, MEK1/MEK2 docking sites swaps, or with empty vector (control). Twenty-four hours post-transfection, cells were transferred to low-serum (0.5%) medium and grown for a further 24 h. Cells were then harvested and ERK1/2 phosphorylation was analyzed by Western blotting. (C) Schematic representation of a MEK2-Ste7 docking fusion protein. Residues 3–14 of MEK2 were replaced with residues 9–17 of Ste7. (D) HEK293 cells were transfected with plasmid constructs encoding constitutively active full-length MEK2-DD, MEK2-DDΔ, MEK2-DDΔ-Ste7, MEK2^{EEAA} or MEK2-Ala. Cells were processed as above and lysates were analyzed by Western blotting.

Fig. 5.

N-terminal location of the MEK2 docking site is not critical for its function. (A) Schematic of the MEK2-DDΔ-Cdock fusion protein. Residues 1–14 of MEK2 were moved to its C-terminus. (B) HEK293 cells were transfected with vectors encoding MEK2-DD, MEK2-DDΔ, MEK2-DDΔ-Cdock or empty vector. Lysates were prepared as described above and were analyzed by Western blotting. (C) Quantification of the levels of phosphorylated ERK1/2. Data represent mean values±standard deviation ($n = 5$).

Fig. 6.

Replacement of the MEK2 docking site with the ERK-binding domain of the Ets-1 transcription factor is able to restore MEK-mediated ERK activation. (A) Schematic diagram of the Ets1-MEK2-DDA fusion protein. Residues 1–14 of MEK2 were replaced with residues 54–138 of Ets-1. (B) HEK293 cells were transfected with MEK2-DD, MEK2-DDΔ, Ets1-MEK2-DDΔ or empty vector. Lysates were prepared and analyzed by Western blotting. (C) Quantification of the levels of phosphorylated ERK1/2. Data represent mean values±standard deviation ($n=5$). (D) HeLa cells were transfected with Ets1-MEK2-DDΔ, MEK2-DD, MEK2-DDΔ or empty vector (control) and their effects on the distribution of endogenous ERK1/2 was examined. Twenty-four hours post-transfection, cells were transferred to low-serum (0.5%) medium and grown for a further 24 h. Cells were then processed as described in Fig. 3. For serum activation (control+), cells were incubated with complete medium (10% serum) for 15 min prior to fixation. (E) HeLa cells were co-transfected with vector encoding ERK2 and either Ets1-MEK2 or MEK2ΔΔ-encoding vectors. Cells were processed as described above. Scale bar=10 μm

Fig. 7.

Replacement of the MEK2 docking site with other docking sites restores MEK –MAPK complex formation in vitro. (A) ^{35}S -labelled MEK2-DD, MEK2-DDΔ, Ets-1-MEK2-DDΔ, MEK2-DDΔ-cdock or MEK2-EEAA proteins were tested for their ability to bind GST-ERK2 (10 μg). Co-sedimented proteins, along with 10% of the original input, were separated by SDS-PAGE and visualized by autoradiography. (B) Relative levels of binding exhibited for each of the MEK-fusion proteins. Results were normalized by setting the amount of full-length MEK2-DD sedimented to 100%. Data shown is the average of three independent experiments with error bars indicating the standard error.

Table 1

Oligonucleotides used in this study

Name	Sequence (5'→3')	Use
LB111	GCGGGATCCACCAT GGCCCAAGAAGAAGCCGA	pcDNA3.1-V5-MEK1
LB112	GCGGGATCCACCAT GGCCCAACCCGGCCCCGACGGC	pcDNA3.1-V5-MEK1Δ
MEK1a	ATACTCGAGGACGCCAGCAGCATGGGTGG	pcDNA3.1-V5-MEK1/MEK1Δ
LB114	GCGAATTCACCAT GCTGGCCCGGAGGAAG	pcDNA3.1-V5-MEK2
LB115	GCGAATTCACCAT GCTGGCCACCATCGCCGAGGGCCCATCC	pcDNA3.1-V5-MEK2Δ
MEK2a	ATACTCGAGCACGGCGGTGCGGTGGGTGT	pcDNA3.1-V5-MEK2/MEK2Δ
MKK3b	GCGTCTAGATGAGTCTTCTCCAGGATCTC	pcDNA3.1-V5-MKK3Δ
MKK3del	ATAGAATTCACCAT GTCTCTGCATGTCCAAGCCA	pcDNA3.1-V5-MKK3Δ
MKK6a	ATAGCTAGCACCAT GTCTCAGTCGAAAGGC	pcDNA3.1-FLAG-MKK6
MKK6b	ATAGGTACCGTCTCCAAGAATCAGTTTAC	pcDNA3.1-FLAG-MKK6
MKK6del	GCGGCTAGCACCAT GGCCAAAAGAAGCATTGAACAACCT	pcDNA3.1-FLAG-MKK6Δ
MEK1 – MEK2ds	ATAGGATCCACCAT GCCTCGGAGGAAGCCGGTGCTCCCCGCGTGACCATCAACCCGGCCCCC	pcDNA3.1-MEK1 – MEK2ds
MEK2 – MEK1ds	GTGGAATTCACCAT GCTGGCCAAGAAGAAGCCGACGCCGATCCAGCTGAACCCTACCATC	pcDNA3.1-MEK2 – MEK1ds
MEK2 – Ste7ds	ATAGAATTCACCAT GCTGGCCAGGAGGAACCTGAAGGGCCTCAACCTCAACCCTACCATC	pcDNA3.1-V5-MEK2 – Ste7ds
MEK2-Ala(1)	<u>AATTCACCAT</u> GCTGGCCGCTGCGGCAGCCGCTGCGGCAGCCGCTGCGGCAGACGT	pcDNA3.1-V5-MEK2-Ala
MEK2-Ala(2)	<u>CTGCCGAGCGGCTGCCGAGCGGCTGCCGAGCGGCCAGCATGGTG</u>	pcDNA3.1-V5-MEK2-Ala
MEK2-CDOCK(1)	<u>CCGGTGGTAGCGGTAGCCGAGGAAGCCGGTGCCGGCGCTCACCATCTGAGTTT</u>	pcDNA3.1-V5-MEK2-CDOCK
MEK2-CDOCK(2)	<u>AAACTCAGATGGTGAGCGCCGGCAGCACCGGCTTCCTCCGGCTACCGCTACCA</u>	pcDNA3.1-V5-MEK2-CDOCK
MEK2-link	ATAGACGTCAACCCTACCATCGCCGAGGGC	pcDNA3.1-V5-MEK2-Ala/Ets1
Ets1a	GCGAAGCTTACCAT GAGTGGTTTCACTAAA	pcDNA3.1-V5-MEK2-Ets1
Ets1b	ATAGACGTCTTTCACATCCTCTTTCTGCAG	pcDNA3.1-V5-MEK2-Ets1
ERK2a	ATAGCTAGCACCAT GGCGGCGGCGGGCGGCG	pcDNA3.1-FLAG-ERK2
ERK2b	GCGGGTACCAGATCTGTATCCTGGCTGGAA	pcDNA3.1-FLAG-ERK2

Where appropriate, restriction sites used for cloning are underlined. Translation initiation or termination codons are highlighted in bold.