ERK5 is targeted to myocyte enhancer factor 2A (MEF2A) through a MAPK docking motif

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One critical component in determining the specificity, and efficiency of MAPK (mitogen-activated protein kinase) substrate phosphorylation is the presence of distinct docking domains in the substrate proteins. Docking domains have been shown to be important for the activities of members of the ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 subfamilies of MAPKs towards their substrates. Here, we demonstrate that docking domains also play an important role in ERK5-mediated substrate phosphorylation. The presence of a docking domain promotes both phosphorylation of myocyte enhancer factor, MEF2A, *in vitro* and its activation *in vivo* by ERK5. Mutational analysis of the MEF2A docking domain demonstrates that the specificity determinants for ERK5 are

similar to those observed with members of the p38 subfamily. A docking domain recognized by ERK5 can direct ERK5 to activate heterologous substrates. Deletion analysis demonstrates that as with other MAPKs, it is the catalytic domain of ERK5 that recognizes the docking domain. Our data therefore extend previous observations on other MAPKs and demonstrate that the requirement for specific docking domains in promoting MAPK action towards substrates is a general property of MAPKs.

Key words: docking motifs, extracellular-signal-regulated kinase 5 (ERK5), mitogen-activated protein kinase (MAPK), myocyte enhancer factor 2 (MEF2), phosphorylation.

INTRODUCTION

Several MAPK (mitogen-activated protein kinase) pathways exist in mammals. The best-studied pathways are the ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 pathways although additional pathways, exemplified by the ERK5 pathway, have been identified that are not functionally grouped with one of these subfamilies (reviewed in [1,2]). Docking interactions amongst members of the ERK, JNK and p38 MAPKs have been identified as important components in their specificity and efficiency of action, and their regulation. These docking interactions involve both the substrates and regulators of MAPKs (reviewed in [3,4,5]). One type of docking site found in transcription factors (referred to as D-domains) is characterized by a region rich in basic amino acids followed by either a LxL motif and/or a triplet of hydrophobic amino acids. These domains are typically less than 20 amino acids long but show only limited sequence similarity. The D-domains are portable and can confer signalling specificity on a heterologous protein towards a particular pathway. D-domains have been identified that permit specific responses to either the ERK, JNK or p38 families or to various combinations of these. However, to date, it is unclear whether other MAPKs, such as ERK5, also use docking domains on their target proteins.

ERK5 differs from other MAPKs due to the presence of an extended C-terminal domain. This C-terminal region acts as a transcriptional activation domain that can activate myocyte enhancer factor (MEF2) transcription factors through interactions with their MADS (MCM1, agamous, *deficiens* and SRF)-MEF2 DNA

binding domain [6,7]. The C-terminal region also contains a nuclear localization signal [8]. The N-terminal region contains the catalytic kinase domain and the phosphorylation site for the upstream activating MEK5 (MAPK/ERK kinase). ERK5 can respond to both mitogenic signals [9] and stress stimuli [10]. c-Myc, SAP-1 (SRF accessory protein 1) and MEF2 subfamily members have been identified as ERK5 substrates [6,11–14]. Different phospho-acceptor sites in MEF2A, -2C and -2D appear to be important for ERK5-mediated phosphorylation and activation. In MEF2A, three sites were shown to be important that are all located in the transcriptional activation domain (TAD) ([15]; residues T304, T312 and S347 in our constructs).

The MEF2 subfamily of MADS-box transcription factors have been shown to be targeted by a subset of p38 MAPKs through docking interactions with the MEF2 D-domain [16]. The D-domain is essential for efficient phosphorylation *in vitro* and activation by the p38 cascade *in vivo*. The structure of the docking domain complexed with p38 has been solved [17], and in combination with mutagenic data [18], a detailed picture has been produced for how the docking domain peptide binds in an extended conformation to the surface of the MAPK. This binding surface is on the opposite side to the catalytic cleft of p38.

In this study we have investigated whether docking interactions with MEF2A are important for its phosphorylation and activation by ERK5. We demonstrate, by deletion and mutagenic analysis and by using chimeric transcription factors, that the D-domain of MEF2 plays an important role in permitting efficient substrate phosphorylation and activation by ERK5. This substrate targeting is mediated by the catalytic domain of ERK5. Thus, in addition

Abbreviations used: CMV, cytomegalovirus; DBD, DNA binding domain; D-domain, MAPK docking domain; Elk-1, Ets-like transcription factor 1; ERK, extracellular-signal-regulated kinase; ERK5-kin, N-terminal catalytic kinase domain of ERK5; ERK5-TAD, ERK5 C-terminal region; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MADS (box), domain found in MCM1, agamous, *deficiens* and SRF; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; MEF2AΔD, MEF2A lacking a D-domain; MEK, MAPK/ERK kinase; MEK5(D), a mutant form of MEK that is constitutively active; S268A etc., substitution of Ser-268 with alanine etc. (denoted by single-letter amino acid coding); SAP-1, SRF accessory protein 1; TAD, transcriptional activation domain; WT, wild-type.

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to the members of the major classes of MAPKs, ERK, JNK and p38, ERK5 also uses docking interactions to facilitate its ability to phosphorylate and activate transcription factor substrates.

MATERIALS AND METHODS

Plasmid constructs

The following plasmids were used for expressing GST (glutathione S-transferase) fusion proteins in *Escherichia coli*. Vectors encoding GST fusions to MEF2A(266–413)(pAS860), GST-MEF2A(283–413)(pAS861), GST-MEF2A(266–413)M2 (pAS886) containing the double mutant L273A/V275A, GST-Elk-1(310–428)(pAS545) (where Elk-1 is the Ets-like transcription factor 1) and GST-MEF2A-Elk-1(pAS865) have been described previously [16]. GST-MEF2A(266–413) with mutations (denoted by single-letter amino acid substitution symbols) S268A (pAS1664); R269A (pAS1665); K270A (pAS1666); P271A (pAS1667); D272A (pAS1668); L273A (pAS1669); R274A (pAS1670); V275A (pAS1671); V276A (pAS1672); I277A (pAS1673); P278A (pAS1674); P279A (pAS1675) were described previously [18].

The following plasmids were used in mammalian cell transfections. pG5E1b reporter construct contains five of the yeast transcription factor, GAL4, DNA binding sites cloned upstream of a minimal promoter element and the firefly luciferase gene [19]. pMEF-Luc contains three copies of the MEF2A binding sites located upstream from a luciferase reporter gene (kindly provided by Dr A. Bannister and Professor T. Kouzarides, Cambridge University, Cambridge, U.K.). Vectors pSR α -HA-ERK5 encoding HA-tagged ERK5, and pSR α -MEK5(D) encoding constitutively active MEK5 were kindly provided by Professor E. Nishida (Kyoto University, Kyoto, Japan) and were described previously [12]. pAS1131 [encoding pCMV (cytomegalovirus)-driven mouse Flag-tagged ERK5 N-terminal amino acids 1–440; ERK5-kin] and pAS1132 (encoding pCMV-driven mouse Flag-tagged ERK5 C-terminal amino acids 512–877; ERK5-TAD) were constructed by inserting *HindIII/BamHI* fragments from pAS1129 and pAS1130 respectively into the same sites in pCDNA3. pAS1129 and pAS1130 were constructed by inserting *BamHI/XbaI* cleaved PCR fragments (primers ADS 955/957 and ADS 956/982) into the same sites of pAS801 and pAS788 respectively. pGAL-c-Jun [encoding GAL4 DNA binding domain (DBD) fused to c-Jun][20], pAS883 (pCMV5-GAL4-MEF2A), pAS884 (pCMV5-GAL4-MEF2A Δ D), pAS900 (encoding GAL4 DBD fused to the Elk-1 TAD), pAS889 and pAS888 (encoding GAL4 DBD fused to the MEF2 docking domain and the c-Jun and Elk-1 TADs respectively) have been described previously [16]. pAS2076 [encoding CMV-driven, Flag-tagged wild-type (WT) full-length MEF2A] and pAS2077 [encoding CMV-driven, Flag-tagged full-length MEF2A(M3); mutations I277A/P278A] were constructed by inserting *KpnI/EcoRI* cleaved fragments from pAS2074 and pAS2075 respectively into the same sites in pCDNA3. pAS2074 and pAS2075 were constructed by inserting *NcoI/EcoRI* cleaved fragments from pAS1203 [16] and pAS2073 respectively into the same sites in pRSETB. pAS2073 was constructed by inserting the *NcoI/XhoI* fragment from pAS1397 [16] into the same sites in pET-nef-PFH. pAS1203 and pAS2073 were also used for expressing His-tagged MEF2A derivatives in *E. coli*.

All plasmids made by using PCR were subjected to automated dideoxy sequencing.

Tissue culture, cell transfection and reporter assays

293 and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine

serum (Gibco BRL). Transfections were performed using Superfect transfection reagent (Qiagen) as described previously [21].

For reporter gene assays a luciferase reporter construct controlled by a GAL4-driven promoter pG5E1bLUC (0.4–1 μ g) was co-transfected with pCMV promoter-driven vectors encoding various GAL4-MEF2A fusion proteins (40–100 ng) and, where indicated, 40–300 ng of MEK5(D) and ERK5 derivatives were transfected.

Transfection efficiencies were normalized by co-transfecting pCH110 plasmid (Pharmacia) and measuring β -galactosidase activities. Cell extracts were prepared and luciferase and β -galactosidase assays were performed as described previously [21].

Protein kinase assays

Recombinant protein kinases, HA-tagged ERK5 and Flag-tagged ERK5-kin, were immunoprecipitated from transfected COS7 cells essentially as described previously, using the appropriate immobilized antibodies [18], except that cells were lysed in 100 μ l triton lysis buffer (Figures 2 and 4). An alternative method of isolating the kinase was also employed for Figures 3 and 5 as described previously [12]. To produce activated ERK5 derivatives, cells were co-transfected with MEK5(D). Protein kinase assays were carried out essentially as described previously (Figures 2 and 4, [18]; Figures 3 and 5, [12]) however, the ERK5 kinase was not eluted from the beads prior to carrying out the kinase reaction. Substrate His-tagged and GST fusion proteins were expressed in *E. coli* BL21 or JM101 and purified as described previously [21].

Western-blot analysis

GAL4-fusion proteins were detected in total 293 cell extracts using anti GAL4 antibody against the N-terminal DNA-binding domain (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Full-length MEF2A derivatives were detected in COS7 cells using an anti-MEF2 antibody (SC10794x, Santa Cruz). Immuno-complexes were detected by using horseradish peroxidase conjugated secondary antibody followed by enhanced chemiluminescence (Pierce).

Figure generation and data quantification

Figures were generated electronically by using Adobe Photodeluxe Business Edition version 1.0 and Powerpoint version 7.0 (Microsoft) software. Data from Western blots are computer generated images (FluorS Max and Quantity One- BIO-RAD). Phosphorimager data from kinase assays were quantified using Quantity One software (BIO-RAD).

RESULTS

Requirement of the D-domain of MEF2A for efficient ERK5-mediated activation of MEF2A

The D-domain of MEF2A has been shown previously to act as a kinase-docking domain and to be important for the efficient phosphorylation of the MEF2A TAD *in vitro* and activation *in vivo* by a subset of p38 MAPKs [16]. MEF2A and the closely related protein MEF2C have also been shown to be targets of the ERK5 pathway [13,14]. Therefore, we wished to examine whether the D-domain of MEF2A played a role in its activation by ERK5.

GAL4 fusion proteins were tested that contained the trans-activation domain of MEF2A, but differed by the inclusion or omission of the upstream D-domain (Figure 1A). In the presence of co-transfected ERK5 and a constitutively active form of its upstream activator MEK5, the transcriptional activity of MEF2A

Figure 1 The D-domain of MEF2A is required for ERK5-mediated transcriptional activation

(A) Diagram illustrating full-length MEF2A and truncated GAL4-MEF2A fusion proteins. The DBD, D-domain (black box), and minimal TAD are shown. The ERK5 phosphorylation sites [15] are indicated (T304 etc.). (B) HEK293 cells were co-transfected with pCMV5 expression vectors encoding GAL4 fusion of either WT or D-domain deletion mutants of MEF2A and MEF2C (0.4 μ g), vectors encoding MEK5(D) (0.1 μ g) and ERK5 (0.3 μ g), GAL4-driven luciferase reporter plasmid (0.4 μ g) and β -galactosidase expression plasmid pCH110 (0.5 μ g). The luciferase activities adjusted to β -galactosidase are presented (means $\pm$ S.E.M., $n = 3$). (C) Expression levels of the GAL4 fusion proteins in the absence and presence of transfected ERK5/MEK5 were examined by Western blotting with an anti-GAL4 antibody.

was greatly enhanced (15-fold; Figure 1B). In contrast, in the absence of the D-domain in MEF2AΔD, this ERK5-mediated activation was severely ablated (2-fold; Figure 1B). Western blotting demonstrated that this differential increase was not due to changes in protein levels, as both wild-type and D-domain deleted versions of MEF2A were moderately enhanced by transfection of ERK5/MEK5 (Figure 1C).

The D-domain of MEF2A is therefore an important determinant of ERK5-mediated activation of its TAD.

Importance of the D-domain of MEF2A for efficient ERK5-mediated phosphorylation of MEF2A

By analogy with other MAPK-mediated activation events, the most likely explanation for the effect of the D-domain on the TAD

Figure 2 Identification of the MEF2A D-domain residues required for targeting of ERK5 by alanine scanning

(A) Diagram illustrating the domain structure of full-length MEF2A and truncated MEF2A proteins fused to GST. The DBD, D-domain (black box), minimal TAD and ERK5 phosphorylation sites are indicated. The sequence of D-domain is shown. Numbers indicate N- and C-terminal amino acids of motifs/domains. (B) Phosphorylation of MEF2A mutants. Kinase assays of WT and mutant GST-MEF2A fusion proteins (5 pmol) with HA-ERK5 were performed for 30 min. An example of a typical kinase assay is shown. The upper band represents autophosphorylated ERK5. The band slightly above GST-MEF2A may be a degraded ERK5 product. Both of these bands are present in the control (without MEF2A) sample (lower panel). A graphic representation of phosphorylation levels is also shown (means $\pm$ S.E.M., $n = 3$; upper panel). The intensity of phosphorylation was standardized to that of the WT (100%), which is represented by the upper dashed line. The level of phosphorylation of the GST-MEF2AΔD is shown by the lower dashed line. The amino acid residues that were mutated into alanine are indicated (single letter coding). M2 refers to the L273A/V275A double mutant.

activity of MEF2A is by directly increasing the ability of ERK5 to phosphorylate its substrate. To establish whether the D-domain of MEF2A plays a direct role in ERK5-mediated phosphorylation, we analysed the ability of ERK5 to phosphorylate GST-MEF2A fusion proteins.

First we compared the ability of ERK5 to phosphorylate GST-MEF2A fusion proteins in the presence and absence of the D-domain. While MEF2A is efficiently phosphorylated by activated ERK5, phosphorylation of MEF2AΔD is severely compromised (Figure 2B, lanes 'M2' and 'ΔD' respectively).

To establish which amino acids were important for recognition by the ERK5 MAPK, we analysed an alanine scanning series across the MEF2A D-domain. With the exception of Asp-272 and Pro-279, all the point mutations resulted in at least a 40% decrease

in phosphorylation by ERK5 (Figure 2B, lanes 1–12). The greatest decrease was seen with Leu-273 and Val-275 mutants that were reduced to virtually the same level as complete deletion of the D-domain (Figure 2B, lanes L273 and V275). The phosphorylation of the M2 mutant (containing the double L273A and V275A mutations) was similarly reduced to basal levels (Figure 2B, lane M2). Leu-273 and Val-275 have been implicated previously as important determinants for p38 MAPK-mediated MEF2A phosphorylation [16,18].

Taken together, therefore, these results demonstrate that the D-domain is an important determinant for phosphorylation of MEF2A by ERK5 and they identify the amino acids in the D-domain important in this process.

The MEF2A D-domain is a portable ERK5-docking motif

One characteristic of MAPK docking domains is that they are portable motifs that can confer ERK5-responsiveness on heterologous substrates. Therefore, we tested the activity of the MEF2A D-domain in the context of two different potential substrates, c-Jun and Elk-1. The docking domain of c-Jun usually specifies this as a JNK substrate [22], whereas the analogous domain in Elk-1 specifies it as a JNK and ERK2 substrate [23].

The MEF2A D-domain was fused to the TAD of c-Jun and Elk-1 in place of their normal MAPK docking domains and the resulting chimeric proteins (Figure 3A) were tested as GAL4 fusion proteins for activation by ERK5 (Figure 3B). Neither c-Jun nor Elk-1 were greatly activated by ERK5 *in vivo* (Figure 3B). The latter observation is consistent with previous data that demonstrated that Elk-1 is not a target of the ERK5 pathway [12]. Fusion of the MEF2 docking domain to the c-Jun TAD resulted in a small increase in ERK5 responsiveness (3-fold; Figure 3B). In contrast, fusion of the MEF2 D-domain to the Elk-1 TAD caused a large increase in ERK5-responsiveness (85-fold; Figure 3B). This enhancement was not due to an increase in the levels of the MEF2-Elk-1 fusion protein (Figure 3B, insert).

To correlate these changes in the transactivation capacity of the Elk-1 TAD with enhanced propensity for phosphorylation by ERK5, analogous GST fusion proteins were tested as substrates for ERK5 *in vitro*. While Elk-1 is a poor ERK5 substrate, the MEF2-Elk-1 fusion protein shows enhanced phosphorylation by ERK5 [Figure 3C, compare lanes 1–4 with 5–8 (upper panel); summarized in graph (lower panel)].

Collectively, these data demonstrate that the MEF2A D-domain can act as a portable docking motif and can confer ERK5-responsiveness on a heterologous substrate.

Figure 3 The MEF2A D-domain acts as a portable ERK5 docking motif

(A) Diagram illustrating GAL4 fusions of c-Jun (shaded boxes) and Elk-1 (white boxes) proteins. GAL4-MEF2-Jun and GAL4-MEF2-Elk1 have the D-domain of MEF2A and C-terminal TAD of Jun and Elk-1 respectively. Numbers indicate N- and C-terminal amino acid residues. Major phospho-acceptor motifs are indicated. (B) ERK5-mediated transcriptional activation by c-Jun, Elk1 and chimeric proteins. HEK293 cells were transfected with expression vectors encoding GAL4 fusion proteins (0.1 μ g), vectors encoding MEK5(D) (0.1 μ g), ERK5 (0.3 μ g), GAL4-driven luciferase reporter plasmid (1 μ g) and β -galactosidase expression plasmid pCH110 (0.5 μ g). The luciferase activities adjusted to β -galactosidase are presented (means $\pm$ S.E.M, $n=3$). The expression levels of the GAL4-MEF2-Elk-1 fusion proteins in the absence and presence of transfected ERK5/MEK5 were examined by Western blotting with an anti-GAL4 antibody (insert). (C) Phosphorylation of GST-Elk-1 and GST-MEF2-Elk-1 fusion proteins by full-length ERK5. Reactions were carried out for the indicated times and 32 P-incorporation determined (top panel) and the amounts of input protein verified by Coomassie staining (bottom panel). The locations of bands corresponding to full-length fusion proteins are indicated by arrows. The data were quantified and shown in the graph (lower panel).

Figure 4 The ERK5 catalytic domain retains targeting activity towards the MEF2 D-domain

(A) Schematic illustration of the domain structure of ERK5 and the truncated proteins, ERK5-kin and ERK5-TAD. The catalytic kinase domain (Kinase) and TAD are shown. (B and C) Reporter gene analysis of ERK5-kin towards MEF2A. (B) COS7 cells were transfected with expression vectors encoding GAL4 fusion proteins (40 ng), MEK5(D) (40 ng), ERK5-kin (120 ng), GAL4-driven luciferase reporter plasmid (0.4 μ g) and β -galactosidase expression plasmid pCH110 (0.2 μ g). The luciferase activities adjusted to β -galactosidase are presented (means $\pm$ S.E.M., $n=3$). (C) As in (B), except that vectors encoding GAL4 fusion proteins (100 ng), MEK5(D) (100 ng), ERK5-kin (0.3 μ g), GAL4-driven luciferase reporter plasmid (1 μ g) and β -galactosidase expression plasmid pCH110 (0.5 μ g) were added in different amounts. Data are presented as activity of each construct in the presence of ERK5 relative to the activity in its absence (taken as 1). (D) Reporter gene analysis of the MEF2A TAD in the presence of the ERK5 TAD. COS7 cells were transfected with expression vectors encoding the indicated GAL4 fusion proteins (40 ng), ERK5-TAD (120 ng), GAL4-driven luciferase reporter plasmid (0.4 μ g) and β -galactosidase expression plasmid pCH110 (0.2 μ g). The luciferase activities adjusted to β -galactosidase are presented relative to the activity of each construct in the absence of transfected ERK5-TAD (taken as 1; means $\pm$ S.E.M., $n=3$). (E) MEF2A phosphorylation by ERK5-kin. Kinase assays of GST-MEF2A and GST-MEF2AΔD fusion proteins (5 pmol) with Flag-tagged ERK5-kin were performed for 30 min. ERK5 was prepared from cells transfected with ERK5-kin alone or in the presence of MEK5(D). A Coomassie-stained version of the same gel is shown below the kinase assay. The location of bands corresponding to MEF2A and ERK5-kin proteins are indicated by arrows.

MAPK docking specificity is mediated by the catalytic domain of ERK5

In contrast to other MAPKs, ERK5 contains a long C-terminal extension to the catalytic domain (Figure 4A). Several activities

have been attributed to this motif, including binding to the DNA binding domain of MEF2A [6]. As other MAPKs use their catalytic domains to bind to the docking domains on substrates, we analysed whether the same was true for ERK5. Therefore, we constructed an expression vector encoding the N-terminal catalytic kinase domain of ERK5 (ERK5-kin; Figure 4A).

First we tested the ability of ERK5-kin to activate the MEF2A TAD *in vivo*. In the absence of co-transfected MEK5, very little effect was seen on the reporter; however, co-transfection of MEK5 and ERK5-kin led to enhancement of the activity of the MEF2A TAD (12-fold; Figure 4B).

To establish the D-domain dependence of this activation, the activity of MEF2AΔD, lacking the D-domain, was analysed. In the absence of the D-domain, no enhancement of the MEF2A TAD was seen by ERK5-kin (Figure 4C). In contrast, the ERK5 C-terminal region (ERK5-TAD) is unable to enhance the activity of the MEF2A TAD, irrespective of the presence of the D-domain (Figure 4D). To confirm that the differences in transactivation we observe are due to a change in the ability of ERK5-kin to phosphorylate MEF2A, we compared the activity of ERK5-kin towards GST-MEF2A fusion proteins *in vitro*. In the absence of the MEF2A D-domain, little phosphorylation of MEF2A was observed. However, phosphorylation of MEF2A by ERK5-kin was greatly enhanced in the presence of the D-domain (Figure 4E, lanes 3 and 4).

Collectively, these data demonstrate that substrate targeting through docking domains is mediated by the catalytic domain of ERK5.

Efficient ERK5-mediated activation of MEF2A-driven gene expression requires the MAPK docking motif

The MAPK docking motif in MEF2A clearly plays an important role in regulating the phosphorylation of the MEF2A TAD and its response to the ERK5 pathway (Figures 1 and 2). To investigate whether the docking motif also plays an important role in the response of the full-length protein to ERK5, we first tested the phosphorylation of WT and a D-domain mutant (M3) versions of MEF2A by ERK5 *in vitro*. In these experiments, we used the M3 mutant version of MEF2A that contains the mutations I277A and P278A. Individually these mutations cause large decreases in phosphorylation by ERK5 in the context of the isolated MEF2A TAD (Figure 2), and together play an important role in p38-mediated phosphorylation of MEF2A [16]. MEF2A(WT) is phosphorylated efficiently by ERK5 after 10 min incubation, however little phosphorylation of MEF2A(M3) is observed, even after 40 min (Figure 5A).

Next, we tested the activity of a reporter gene driven by MEF2 binding sites in the presence of full-length versions of WT and mutant MEF2A (Figure 5B). The MEF2-driven reporter gene was efficiently activated in the presence of WT MEF2A and activated ERK5. However, in the presence of the mutant version of MEF2A, MEF2A(M3), the activity of the reporter in response to activated ERK5 was much reduced (14% of WT protein). This difference was not due to differing amounts of MEF2A protein in the cells (Figure 5B, bottom panel). Thus, the MAPK docking domain in MEF2A plays an important role in the response of MEF2A to the ERK5 pathway *in vivo* when investigating either the isolated transcriptional activation domain or the full-length protein.

DISCUSSION

Docking domains play a major role in dictating the specificity of substrate phosphorylation by MAPKs [3,4,5]. Here we

Figure 5 The MEF2A D-domain plays an important role in the response of MEF2-dependent genes to ERK5 activation

(A) Phosphorylation of WT and docking domain mutant (M3) full-length MEF2A proteins by ERK5. Reactions were carried out for the indicated times and 32 P-incorporation was determined (top panel), and the amounts of input protein verified by Coomassie staining (bottom panel). The locations of bands corresponding to full-length MEF2A are indicated by arrows. The band corresponding to autophosphorylated ERK5 is also indicated in the top panel. (B) A schematic of the MEF2-luciferase reporter gene is shown (top panel). Luciferase reporter gene assays were carried out on the MEF2-luciferase reporter (0.4 μ g) in COS7 cells in the presence of the indicated cotransfected plasmids MEF2A(WT) (80 ng), MEF2A(M3) (80 ng), ERK5 (120 ng) and MEK5(D) (40 ng). The relative luciferase activities adjusted to β -galactosidase are presented (means $\pm$ S.E.M., $n = 3$; middle panel). Western blots are shown (bottom panel) of the levels of MEF2A (WT, lanes 1 and 2 and M3, lanes 3 and 4) under the indicated conditions. (C) Model for ERK5-mediated activation of MEF2A. Interactions between the C-terminus of ERK5 and the N-terminal region of MEF2A result in recruitment of a TAD from ERK5 to MEF2A. Additional

demonstrate that in addition to members of the major classes of MAPKs, ERK, JNK and p38, ERK5 also uses docking interactions to facilitate its ability to phosphorylate and activate transcription factor substrates.

ERK5 has previously been shown to bind to MEF2 transcription factors through interactions between the MADS-MEF2 DNA binding domain and the C-terminal extension in ERK5 [6,7]. This interaction is thought to bring the TAD located in the C-terminus of ERK5 into a DNA-bound complex with MEF2 proteins and hence activate transcription. However, this interaction appears not to affect MEF2 phosphorylation itself [7]. Here we have shown that the kinase domain of ERK5 is targeted to the D-domain of MEF2A. This interaction is clearly important in enhancing the ability of ERK5 to potentiate the activity of the MEF2A TAD in response to ERK5 pathway activation (Figures 1B, 4B and 4C). Mutational analysis of the D-domain in the context of full-length MEF2A also reduces its transcriptional activation response to ERK5 activation (Figure 5B). This enhancement is mirrored by the observation that the MEF2A D-domain is also required for efficient phosphorylation of the TAD (Figure 2B and 4E) and the full-length protein (Figure 5A) by ERK5 *in vitro*. Thus a model emerges in which ERK5 can potentially interact with two different regions of MEF2A (Figure 5C). Interactions between the C-terminal end of ERK5 and the N-terminus of MEF2A can cause transcriptional enhancement of MEF2 activity by recruitment of the ERK5 TAD. Further interactions between the kinase domain of ERK5 and the D-domain of MEF2A can then lead to phosphorylation and activation of the MEF2A TAD in a signal-dependent manner. Together, this permits a high level of activation to be achieved through MEF2A via the ERK5 pathway.

Our data further extend the paradigm that docking domains play an important role in mediating the downstream effects of MAPK signalling. Previous studies on MEF2A demonstrated that two p38 family members, p38 α and p38 β_2 , are specifically targeted to MEF2A through the D-domain [16]. Alanine scanning revealed the residues in MEF2A that are important in this process [18] and structural studies confirmed this mutagenic analysis [17]. A comparison of the alanine scanning data for ERK5-mediated MEF2A phosphorylation (Figure 2B) and previous data with p38 MAPKs, demonstrates an almost identical profile. In particular, Leu-273 and Val-275 were particularly sensitive to mutation in both cases. Thus, the docking requirements for a subset of p38 MAPKs and ERK5 appear very similar. This suggests that the interaction surface on the kinase is also likely to be similar. Indeed, a comparison of the residues located at the analogous positions in ERK5 to those in p38 α that form the docking groove for MEF2A [17] reveals that of the 15 residues involved, seven are identical and seven are highly conserved between the two proteins. Consistent with this conclusion is the observation that SAP-1 is also a p38 and ERK5 substrate. Substrate recognition in this case is also via a docking domain that is recognized specifically by the p38 α and p38 β_2 MAPKs [24]. It remains to be seen how far this overlap between substrate specificity through MAPK docking domains extends.

Neither Elk-1 (Figure 3A; [12]) nor c-Jun (Figure 3A) represent ERK5 targets *in vivo*. However, by inserting the MEF2 docking domain in place of the natural docking domains of these substrates, ERK5 responsiveness could be conferred on these transcription factors (Figure 3). However, while MEF2 D-domain

interactions between the N-terminal catalytic domain of ERK5 and the D-domain (shown by a black box) of MEF2A lead to phosphorylation (indicated by 'P') of the MEF2A TAD and enhanced transcriptional activation. Together this can lead to a bipartite activation process of MEF2A.

fusions to Elk-1 were efficiently activated by ERK5, the analogous fusions to c-Jun were only slightly responsive. Thus, in addition to the docking interaction, the local context of the phospho-acceptor motifs clearly plays an important part in the response of transcription factors to ERK5.

In summary, our data further extend the paradigm of MAPK docking domains being important for generating substrate specificity to ERK5. It appears likely that other MAPKs, not studied to date, will also use such interactions.

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