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Analysis of mitogen-activated protein kinase activation and interactions with regulators and substrates

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Abstract

Mitogen-activated protein kinase (MAPK) cascades are ubiquitous signal transduction modules in eukaryotes that are of great interest and importance. Here, we summarize some useful methods for the analysis of MAPK signaling, including methods to (1) detect MAPK activation in cells, with an emphasis on using phosphorylation-state-specific antibodies raised against mammalian phosphopeptide sequences to detect the activation of MAPKs in other species; (2) estimate the cellular concentrations of MAPKs and other proteins of interest; (3) detect and quantify the stable physical association of MAPKs with their substrates and regulators, and estimate the relevant dissociation constants; (4) delineate the MAPK-binding regions or domains of MAPK-interacting proteins, with particular emphasis on the identification and verification of MAPK-docking sites. These procedures are broadly applicable to many organisms, including both yeast and mammalian cells.

Keywords

MAP kinase; Phosphorylation; Protein–protein binding; Protein–protein interaction; Docking sites; Phosphorylation-state-specific antibodies; Dissociation constant

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades are found in almost all eukaryotic organisms, and are expressed in virtually all tissues. MAPK cascades contribute to the regulation of diverse responses, including normal and pathological aspects of cell growth, division, differentiation, and death [1,2]. The MAPK cascade is a set of three sequentially acting protein kinases. Starting from the bottom and working back up, there is a MAPK (also termed extracellular-signal-regulated kinase, or ERK), which is phosphorylated and thereby activated by a MAPK/ERK kinase (MEK, MAPKK, MAP2K, or MKK). MEK activity is regulated, in turn, via phosphorylation by the top-most member of the module, a MEK kinase (MAP3K or MEKK) [3]. Activated MAPKs phosphorylate targets including transcription factors, other kinases, and other enzymes [4,5]. Scaffold proteins often assist in MAPK activation by binding to the MAPKs and other components of the cascade [6,7]. Finally, activated MAPKs are dephosphorylated and thereby deactivated by a battery of protein phosphatases [8–11].

Mammals contain four major MAPK cascades, designated the ERK1/2, ERK5, p38 and JNK cascades after their MAPK components. Most eukaryotes that have been examined to date appear to contain probable orthologs of p38 and ERK1/2. For example, in the yeast

Saccharomyces cerevisiae, Hog1^{MAPK} is orthologous to p38, and Kss1^{MAPK} and Fus3^{MAPK} are likely orthologs of ERK1/2 [12]. The JNK pathway appears to have arisen after the fungal-animal split [12]. The origin of the ERK5 pathway is uncertain at present.

MAP kinases are regulated by MEK-mediated phosphorylation of their activation loop, a region of the catalytic domain located between conserved kinase subdomains VII and VIII in the primary structure, just below the catalytic cleft in the tertiary structure [13]. MEKs/MKKs phosphorylate both a tyrosine and a threonine in a TXY motif found in the MAPK activation loop; for this reason, MEKs are called “dual-specificity” kinases [14]. Phosphorylation of the activation loop induces it to refold, causing subtle conformational changes which reverberate through the rest of the enzyme [15]. For example, in the ERK2 MAPK, activation loop phosphorylation unblocks the active site and promotes a closure of the upper and lower lobes of the kinase domain that brings the catalytic residues into their correct orientation [16]. As a result, the catalytic rate of ERK2 is increased by over 5000-fold [15].

MAPKs bind to many of their substrates and regulators in a relatively stable, relatively high-affinity fashion (‘relatively stable’ implies a half-life on the order of seconds to minutes, and ‘relatively high-affinity’ implies a K_d in the low micromolar range or lower). This physical association is often very important for efficient regulatory transactions such as phosphorylation, dephosphorylation or subcellular localization. Often, such interactions are mediated by MAPK-docking sites—short amino acid stretches that are located on MAPK-interacting proteins [17,18] and that bind to one or more of several characterized cognate binding regions on MAPKs [19,20].

In this paper, we summarize methods we have used successfully and repeatedly to detect MAPK activation in cells [21–24], estimate the cellular concentrations of MAPKs and other proteins of interest [25]; detect and quantify the stable physical association of MAPKs with their substrates and regulators [24–32], and delineate the MAPK-binding regions or domains of MAPK-interacting proteins [24–26,29–32].

2. Buffers and materials

The following reagents are used in the protocols described herein:

2.1. Buffers

2.1.1. Lysis/binding buffer—Lysis/binding buffer is 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 125 mM potassium acetate, 12.5% (v/v) glycerol, 0.1% (v/v) Tween 20, and 1 mM DTT (added fresh from a 1 M stock solution stored at –20 °C). This buffer can be used for extract preparation, and for both the incubation and wash steps of binding assays. A reasonable alternative buffer is phosphate-buffered saline that has been raised to 12.5% (v/v) glycerol, 0.1% (v/v) Tween 20, and 1 mM DTT.

2.1.2. Buffer additions—The following additions to the above lysis/binding buffers are recommended depending on the application:

1. If cell extracts are involved, protease inhibitor cocktail should be added. These cocktails are available from commercial suppliers such as Sigma and are available in special formulations for bacteria, fungi, mammalian cells, etc. We use Sigma protease inhibitor cocktail for fungi (catalog number P-8215), 0.4% (v/v).
2. If the goal is to measure phosphorylation state on an immunoblot, phosphatase inhibitor cocktail may be added. Again, these cocktails are commercially available from various suppliers. We use Sigma phosphatase inhibitor cocktail 1 (Catalog

No. P-2850), 1% (v/v), plus Sigma phosphatase inhibitor cocktail 2 (P-5726), 1% (v/v). Since some of the chemicals in such cocktails may inhibit protein kinases as well as phosphatases, we generally use a simpler cocktail consisting of 25 mM β -glycerophosphate and 1 mM sodium orthovanadate if the goal is a biochemical assay such as an *in vitro* kinase reaction.

3. If a glutathione-*S*-transferase (GST)-pull down assay is being performed, the DTT is raised to 5 mM (as recommended in [33]), and the binding buffer is supplemented with 0.1% (1 mg/ml) bovine serum albumin (BSA). These additions are made to the buffer used in the binding reaction itself, and not to the wash buffer.

2.1.3. 3× SDS sample buffer—3× SDS sample buffer is 60 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 1% (w/v) DTT (Note. 1 M DTT = 15%), 20% (v/v) glycerol, and 0.001% (w/v) bromphenol blue. This buffer is stored frozen in 1 ml aliquots.

2.1.4. 1× High pH SDS sample buffer—1× High pH SDS sample buffer is 60 mM Tris-HCl, pH 8.8, 2% (w/v) SDS, 0.3% (w/v) DTT, 7% (v/v) glycerol, and 0.001% (w/v) bromphenol blue. This buffer is stored frozen in 1 ml aliquots.

2.2. Antisera

For detecting cross-species-reactivity with phospho-specific antibodies, we have found that polyclonal antibodies generally work better than monoclonal. An anti-phospho-ERK antibody we have had success with is “Phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody” (Catalog No. 9101) from Cell Signaling Technology. An anti-phospho-p38 antibody we have had success with is “Phospho-p38 MAP kinase (Thr180/Tyr182) antibody” (Catalog No. 9211), also from Cell Signaling Technology.

3. Preparation of a protein extract

To detect MAPK activation by immunoblotting or immune-complex kinase assay, or to study MAPK protein-protein interactions by co-immunoprecipitation or co-sedimentation from cells, it is first necessary make a protein extract by lysing the cells of interest in an appropriate buffer (such as the lysis/binding buffer described in Section 2). How the cells are lysed will depend upon the specific cell type and organism, but some general principles apply:

1. It is preferable to freeze the cells before lysis, rather than to freeze the extract after lysis. Thus, the day's work will often begin with the thawing of a frozen cell pellet.
2. Use enough cells in a small enough volume of buffer to get a relatively concentrated extract (approx. 5–10 mg protein/ml buffer).
3. Keep the cells and the extract very cold during preparation. An ice water bath works well for this purpose.
4. Spin the extract to remove insoluble debris, but do not spin too hard or too long. A 5 min spin in a microfuge (~13,000g) is fine, but 100,000g may pellet your MAPK.
5. Carefully measure the protein concentration in the extract. Standard methods, such as the Bradford assay, compare the absorbance of a sample of dye-bound extract to the absorbance of a standard curve of known amounts of dye-bound BSA.
 - a. Typically, 10–20 μ g of protein will be in the linear range of the standard curve. This would correspond to 1–2 μ L of concentrated extract. Therefore, to avoid error caused by pipetting small volumes of the

concentrated extract, dilute a small portion of extract 1:10 to use for measuring.

4.2. TCA precipitation of proteins

Trichloroacetic acid (TCA) precipitation is useful for concentrating proteins. It can also in some cases result in a cleaner sample, as certain cellular components that degrade the quality of SDS-PAGE and immunoblot analysis will remain in the supernatant. TCA works by acid denaturation of proteins, so this method should only be used to concentrate and clean up protein samples prior to immunoblotting. Note. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

1. Bring the protein samples to 1 $\mu\text{g}/\mu\text{L}$ by adding appropriate buffer (e.g. lysis/binding buffer + protease and phosphatase inhibitors). For example, to precipitate 250 μg , bring samples to 250 μL . Place samples in an ice-water bath.
2. Add 1/10 volume 100% (w/v) trichloroacetic acid (TCA) to the samples. Gently, yet thoroughly, vortex.
3. Incubate in ice water 10 min.
4. Spin 5 min at 4 °C in a microfuge.
5. Remove supernatant completely, then wash pellet in 500 μL acetone (careful—pellet may become fluffy and fragmented). Remove the acetone completely.
6. Air dry in 37 °C incubator and resuspend samples in 1/10 original volume of 1 $\times$ high pH SDS sample buffer. Pellets may require heating (70–90 °C, 3 min) and vortexing to get into solution. If the sample buffer turns yellow then the pH is too low, and the pellet will not resuspend well. Once resuspended, the protein will be at a concentration of 10 $\mu\text{g}/\text{L}$, assuming no losses during precipitation.
7. Before loading the samples on a gel, spin 2 min in microfuge to pellet any material that did not resuspend.

4.3. Alternative methods

Kinase activity—Perhaps the most reliable way to measure MAPK activation is to monitor the kinase activity by immunoprecipitating the MAPK of interest from cell extracts and assessing its ability to phosphorylate a suitable substrate in an *in vitro* reaction. This is a standard procedure that will not be further discussed here [37].

Gel shift—Some MAPKs exhibit a detectable shift in their electrophoretic mobility upon phosphorylation, which can be used in lieu of anti-phospho-antibodies to monitor their activation state. An excellent example is *Xenopus* p42^{MAPK} [38].

In gel kinase assay—This procedure is described elsewhere [39].

5. Estimating the cellular concentration of MAPKs and other proteins

5.1. Summary of the method

To estimate the cellular concentration of a given protein X requires (1) a standard containing a known concentration of protein X, and (2) a whole cell extract for which the total protein concentration has been accurately measured. A dilution series of the standard and a dilution series of the sample are then run on the same SDS-PAGE and subjected to immunoblotting, or perhaps, given a clean enough antibody, compared by ELISA. This exercise will determine the weight of protein X present in a given weight of total cell protein.

The standard is purified from bacteria or some other source, or produced by *in vitro* transcription and translation. This latter option requires the ability to accurately determine the yield of a protein translated *in vitro*. A method for this is given below.

5.2. Related methods

Recently, a similar method has been used to measure cellular protein concentration on a genomic scale in yeast [40]. This was accomplished by semi-quantitative immunoblotting of extracts prepared from over 4000 different yeast strains, each expressing a different tandem-affinity-purification (TAP)-tagged-yeast protein from the endogenous promoter. A control TAP-tagged protein was used as the standard. This method may be prone to some bias for fusion proteins that do not transfer to the immunoblot membrane as efficiently as the standard; this would lead to an underestimation of the cellular concentrations of poorly transferring proteins. Nevertheless, for Ste7^{MEK}, Fus3^{MAPK} and Kss1^{MAPK}, this method gave estimates in good agreement with those obtained using native test proteins and standards produced by *in vitro* translation [25].

5.3. Example calculation

Suppose we desire to calculate the cellular abundance of a protein called X. We run a dilution series of our standard of X on the same gel as a dilution series of extract from the cells of interest. Suppose the lane containing 10 ng of protein X standard gives the same intensity staining pattern as the amount of protein X in 10 µg of total cell protein. This indicates that protein X constitutes 0.1% of total cell protein. It has been estimated that the average yeast cell contains 6 pg of total protein. Hence, there are 6 fg of protein X per cell. If protein X has a mass of 50 kDa, then protein X weighs $\sim 10^{-19}$ g ($50,000 \text{ g/mole} / 6.023 \times 10^{23} \text{ molecules/mole} = \sim 10^{-19} \text{ g/molecule}$). Hence the total number of molecules of protein X per cell is 60,000 ($6 \times 10^{-15} \text{ g/cell} / 10^{-19} \text{ g/molecule} = 6 \times 10^4 \text{ molecules/cell} = 100 \text{ zmoles/cell}$). For mammalian cells, perform the same calculations assuming 50 pg protein/cell. To convert to concentrations, assume a volume of 0.1 pL for a yeast cell and 0.5–1 pL for a typical mammalian cell. Thus, in yeast, 60,000 molecules/cell corresponds to a cellular concentration of approximately 1 µM.

5.4. In vitro transcription and translation

In vitro translation is a useful method to produce protein standards for estimating cellular concentrations, and to produce radiolabeled protein for use in quantitative binding assays. Kits that enable the coupled *in vitro* transcription and translation of plasmid- or PCR-product-encoded proteins are available from manufacturers such as Novagen or Promega. Some of the advantages and disadvantages of this method are as follows:

Advantages

1. *In vitro* transcription/translation is probably the fastest and easiest way to move from a cloned open reading frame (ORF) to a protein in a test tube. All that is required is that the ORF be subcloned into a vector containing a promoter for SP6 or T7 RNA polymerase, or that one of these promoters is included in a PCR primer that is used to amplify the ORF.
2. Proteins that cannot be expressed in soluble form in bacteria are generally perfectly soluble when produced by *in vitro* translation.
3. The protein of interest can be radiolabelled by the inclusion of [³⁵S]methionine in the translation reaction. The protein is produced in radiopure form, meaning that it is the only protein that gets significantly labeled.

Disadvantages

1. The protein of interest is produced in small amounts, e.g. about 1–5 ng/μl, which, for an average size protein of 50 kDa, converts to 20–100 nM. A procedure to increase yield has recently been developed, however [41].
2. The protein is produced in a cell lysate that is full of other proteins, tRNAs, amino acids, ribonucleotides, etc. These biomolecules may interfere with downstream applications. The *in vitro* translated protein will typically represent about ~1/20,000 (by weight) of the total protein in the translation reaction. A simple ammonium sulfate precipitation procedure has been described that results in a >20-fold purification of *in vitro*-translated protein relative to lysate-derived protein [42], and is detailed below. Use of this procedure can minimize band distortion caused by overloading of gel slots.
3. There is often a significant production of fragments of the protein of interest. This is due to the combined effects of premature translation termination and internal initiation.

5.5. Quantifying the yield of *in vitro* translation

1. Estimate the endogenous concentration of methionine in the lysate being used. Typically, lysates for *in vitro* translation are supplemented with an amino acid cocktail lacking methionine, so as to maximize the efficiency of labeling with added radioactive methionine. However, there is still the endogenous concentration of methionine to account for. Typical rabbit reticulocyte lysate preparations contain between 5 and 15 μM endogenous methionine. Compared to this, any added [³⁵S]methionine is essentially just a tracer. To estimate the endogenous concentration of methionine in a given batch of extract, compare the percent incorporation of [³⁵S]methionine into test protein in lysate samples supplemented with 0, 5, 10 and 15 μM cold methionine. When the percent incorporation of the tracer is reduced by 50%, the concentration of added methionine is equal to the endogenous concentration of methionine.
2. Translate the protein of interest and determine the percent incorporation of [³⁵S]methionine into protein by TCA precipitating a small aliquot of the completed translation reaction onto a filter disk.
3. Use the information obtained in steps 1 and 2 to calculate the concentration of methionine incorporated into protein. This number, divided by the number of methionines in the translated protein, gives the concentration of protein molecules produced. An example of such calculations is shown in Table 1.

In our experience, using kits based on rabbit reticulocyte lysate, typical protein yields are 20–100 fmoles/μL, that is, 20–100 nM. For an average size protein of 50 kDa, this converts into a weight per volume of 1–5 ng/μL. We typically use 1 pmole of *in vitro*-translated protein in a 200 μL binding reaction. This typically requires the addition of between 10 and 50 μL of translated lysate.

5.6. Ammonium sulfate precipitation of *in vitro* translation reactions

1. After the translation reactions are finished, add an equal volume of 4 M ammonium sulfate/40 mM Tris/1 mM EDTA, pH 7.5, to each translation reaction.
2. Chill samples in an ice water bath 15 min.
3. Spin 15 min at 4 °C in microfuge.
4. Remove supernatant (discard in radioactive waste if necessary).

5. Add 3× original volume of cold 2 M ammonium sulfate/20 mM Tris/0.5 mM EDTA/1 mM DTT, pH 7.5. Try not to disturb the pellet.
6. Spin briefly and remove supernatant, then pulse again and remove last traces of liquid.
7. Store here on ice in cold room, or store frozen at -80°C , or resuspend directly in original volume of buffer of choice.

6. Detecting the interaction of MAPKs with other proteins

6.1. Summary of the methods

There are a wide variety of methods for detecting protein–protein interactions [43], most of which are suitable for analyzing the interactions of MAPKs with their substrates and regulators. In our work, we find ourselves often using one of the following methods:

1. Co-sedimentation of *in vitro*-translated test proteins (the fish) with glutathione-S-transferase (GST) fusion proteins (the bait). This method is commonly referred to as a “GST pull-down” [44].
2. Co-immunoprecipitation of two proteins from cell extracts. Here, the protein to which the antibody binds is the bait and the co-immunoprecipitating protein is the fish.
3. The yeast two-hybrid assay [45,46].

All three methods are well established, with many published protocols. Here, we present a procedure for estimating dissociation constants from the results of binding assays, and give some advice for using co-immunoprecipitation from cell extracts to examine MAPK-mediated protein–protein interactions. Information on the yeast two-hybrid assay is available elsewhere [45,46].

6.2. Estimation of dissociation constants from the results of binding assays

The dissociation constant, or K_d , is a number that represents how strong the interaction between two ligands is: the lower the K_d , the stronger the interaction (K_d and free energy are directly interconvertible). Knowing the K_d allows an informed comparison of the relative strength of binding of different ligands. We present this method using the example of an *in vitro*-translated fish protein co-sedimenting with a GST fusion bait protein, but it is readily applicable to other types of binding assays, e.g. co-immunoprecipitation. The essence of the method is that dissociation constants can be calculated from the known input concentrations of both the bait and fish, and a determination of the amount of bait–fish complex formed.

For a simple bimolecular binding reaction, the relevant chemical equation is $A + B \rightleftharpoons AB$. Here, A is the ^{35}S -labeled protein, and B is the GST fusion protein. Then

$$K_d = ([A]_{\text{eq}} \times [B]_{\text{eq}}) / [AB]_{\text{eq}}, \quad \text{where}$$

$$[A]_{\text{eq}} = ([A]_0 - [AB]_{\text{eq}}),$$

$$[B]_{\text{eq}} = ([B]_0 - [AB]_{\text{eq}}),$$

and $[A]_0$ and $[B]_0$ are the input concentrations of A and B. $[AB]_{\text{eq}}$ is the percentage of A that co-sediments with B (readily determined if A is radiolabelled) multiplied by $[A]_0$. This number must be corrected, if necessary, for the efficiency of sedimentation/precipitation of the bait. The bait sedimentation efficiency can be assumed to be 100% if bead-bound GST fusion protein was added to the binding reactions, but is likely to be substantially less than

100% if eluted GST fusion proteins were added to the binding reaction, and then and glutathione sepharose or an antibody/bead combination was added to promote sedimentation/precipitation.

K_d estimates should be calculated by applying the above formula to the results of multiple replicate experiments and averaging the results [25,30]. An example is shown in Table 2. As is true of most activity calculations, the resulting K_d estimate is based on the assumption was that 100% of input molecules are active (i.e. capable of binding).

Clearly, accurate determination of protein concentrations is necessary to achieve reliable estimates. GST-fusion proteins bound to glutathione–Sepharose beads can be quantified by comparison to known amounts of BSA on a Coomassie blue-stained protein gel. Eluted GST fusions can be quantified by a standard Bradford assay. As indicated above, it is quite convenient to have the fish protein be radiolabelled (e.g. as a result of *in vitro* translation), in which case its concentration can be determined by the method given in Section 5.5.

Examples of GST-pull down assays involving MAPKs that were quantified using the above technique have been described [24,28–30,32]. In these studies, GST–MAPKs were used to pull down *in vitro*-translated MEKs or substrates, or GST-fusions to portions of MEKs, substrates or scaffold proteins were used to pull down *in vitro*-translated MAPKs.

6.3. Co-immunoprecipitation of MAPKs and their binding partners from cell extracts

Typical dissociation constants for the interaction of MAPKs with substrates and regulators is in the range of 5–200 μ M [28–30]. Practically, this means that these interactions are somewhat difficult to detect by co-immunoprecipitation. Success is critically dependent on the use of a sufficiently high concentration of extract protein in the reaction. For both yeast and mammalian cell applications, a protein concentration of 5 mg/ml is recommended as a starting point (i.e. 1 mg total cell protein in a reaction volume of 200 μ L); if this works, the use of less protein can be attempted during optimization. In addition, overexpression of both the MAPK and its binding partner greatly increases the chances of success. An example of a successful co-immunoprecipitation experiment can be found in Molina et al. [32]. In this study, the ERK2 substrate microphthalmia-associated transcription factor (MITF; bait) and ERK2 (fish) were co-immunoprecipitated from human kidney epithelial (HEK293) cells.

7. Delineation of MAPK-binding domains or regions

7.1. MAPK-docking sites on substrates and regulators

The ability of MAPKs to effectively and specifically recognize their substrates is enhanced by their capacity to bind to those substrates with relatively high affinity. Often, this interaction occurs via MAPK-docking sites on substrate proteins [17,18]. These docking sites are small regions within substrates that bind with relatively high affinity to MAPKs, in some cases showing specificity for particular MAPKs. Docking sites are also found on MAPK regulators such as MEKs, phosphatases and scaffold proteins.

One class of MAPK-docking site, designated the ‘D-site’, has the consensus (K/R)_{1–3}–X_{1–5}– ϕ X ϕ , where ϕ is a hydrophobic residue, typically Leu, Ile, Val or Met. D-sites (also called ‘D-domains’, ‘DEJL motifs’, or ‘kinase interaction motifs’) were first identified in the yeast MEK Ste7 and the transcription factors c-Jun and Elk-1 [25,47–49], and were subsequently found in numerous MAPK-interacting proteins. A second docking motif (consensus L–X_{1–2}–(R/K)_{2–5}), related to the D-site, is found in MAPK-activated kinases such as RSK1 [50]. Another docking motif for MAP kinases (consensus FXFP) has been named the ‘DEF motif’ [51]. Fig. 3 shows examples of selected MAPK docking sites culled from human and yeast proteins.

MAPK-docking sites are typically found near (within 50–100 residues of) the target phosphorylation site(s) on substrate proteins. For example, the JNK2 MAPK binds to a D-site on c-Jun located within residues 30–45, and phosphorylates c-Jun on Ser63 and Ser73 [49]. A single docking site can enhance the phosphorylation of multiple target sites, although some substrates contain multiple MAPK docking sites, each of which may direct the MAPK to phosphorylate a specific subset of residues [52]. Fig. 4 is a cartoon that shows one way to visualize how docking sites work.

MAPKs are proline-directed serine/threonine kinases, thus they phosphorylate the Ser or Thr in the dipeptide motif S/T-P. Leu is a preferred residue at the –1 position, and Pro is preferred at –2 (or, less so, at –3); thus, the optimal site is P-L-S/T-P [53]. Many physiological target sites, however, do not match the ‘optimal’ consensus.

7.2. Cognate docking sites on MAPKs

Structural and mutagenesis studies indicate that the basic submotif of the D-site contacts two closely spaced acidic patches on the MAPK surface—the ‘CD/sevenmaker’ region and the ‘ED region’ [24,54–58]. The hydrophobic submotif of the D-site binds to a ‘hydrophobic docking groove’ located close the CD/ED regions [54,56,57,59].

The L-X_{1–2}-(R/K)_{2–5} motif also makes contacts with the CD/sevenmaker and ED regions [55,58]. It is not known where the hydrophobic portion of this motif binds.

In contrast, the DEF motif binds to a different hydrophobic pocket (i.e. distinct from the one that the D-site binds to). This pocket is located below the MAPK active site, and is occluded in unphosphorylated ERK2 [60]; thus, the DEF motif preferentially binds to the dually phosphorylated, activated isoform of ERK2 [60].

7.3. Other MAPK-binding domains

Although many MAPK-interacting proteins contain one or more of the MAPK dockings sites described above, there are also numerous MAPK interacting proteins that apparently do not contain one of these well-recognized, commonly used MAPK docking sites. Examples include MITF [32], yeast Ste12, which binds preferentially to unphosphorylated Kss1^{MAPK} [26], and Ets-1, which binds to ERK2 via its pointed domain [61]. Of course, it is possible that some of these proteins will turn out to bind to the same regions of MAPKs that the more widely utilized docking sites interact with.

7.4. Delineating MAPK-binding domains or region of MAPK-interacting proteins

Presuming that a novel MAPK-interacting protein has been identified, a standard technique for localizing the particular domain or region that is responsible for MAPK binding is to produce a series of protein fragments and test them for MAPK binding. This is facilitated by making the fragments by *in vitro* translation. An example of such a procedure can be found in Bardwell et al. [25] and Molina et al. [32]. In the former example, the use of progressively smaller N-terminal fragments of Ste7^{MEK} eventually led the authors to identify a high-affinity D-site at the very N-terminus of Ste7 [25]. In the latter case, it was concluded that the MAPK-binding domain of MITF constitutes approximately 100 residues and does not contain any obvious MAPK-docking motifs [32].

7.5. Identification of MAPK-docking sites

At present, the best way to identify putative MAPK docking sites in a sequence of interest is to scan the sequence by eye or with a simple pattern matching algorithm. The program Scansite [62] also contains probability weight matrixes for the identification of D-sites and DEF motifs.

7.6. Experimental verification of MAPK docking sites

How can a putative MAPK-docking site that has been identified in a MAPK-binding protein be verified? Some of the experimental results obtained with bona fide MAPK docking sites are:

1. Deletion of the docking site, or mutation of critical conserved residues in the docking site, results in diminished MAPK binding [29,30].
2. Likewise, such mutations decrease the efficiency of MAPK-dependent enzymatic reactions. Substrates with crippled docking sites are phosphorylated less efficiently by their cognate MAPKs [48]. MEKs lacking docking sites exhibit greatly reduced ability to activate their cognate MAPKs [21].
3. The docking site sequences, fused to GST or anchored to a membrane, are often sufficient to bind to MAPKs [24,29,30].
4. Short docking-site-containing peptides bind to their cognate MAPK and inhibit the ability of the MAPK to bind to certain proteins, phosphorylate certain substrates and be dephosphorylated by certain phosphatases (in theory, those binding partners containing the same class of docking site) [27,30].

Potentially complicating such analysis is the possibility that the MAPK-binding protein of interest may contain multiple docking sites. For example, Elk-1 [51] and MKP-1 [63] contain both a D-site and an FXFP/DEF site, Net contains two D-sites (one for JNK, one for ERK) as well as a putative FXFP/DEF site [64], and MKK7 contains three low-affinity D-sites that interact to create a relatively high-affinity MAPK-docking platform [31].

7.7. Affinity of MAPK-docking sites

The binding affinity of isolated D-site peptides for their cognate MAPKs, as well the affinity of D-site-dependent binding interactions involving larger polypeptides, have been measured in several publications by several methods. The D-site of yeast Ste7 has an exceptionally high-affinity for the MAP kinases Fus3 and Kss1, with a K_d of 5–100 nM [25,29,59]. D-sites found in mammalian MAPK-binding partners typically have K_d s in the low micromolar range [28,30,31], e.g. ~5 μ M for MEK2-ERK2 [29], ~30 μ M for MKK4-JNK3 [30] (see Table 2). A particularly strong interaction is that between the D-site of the JIP-1 scaffold protein and JNK MAP kinases (apparent K_d <1 μ M) [65]. To our knowledge, the binding affinities of other classes of MAPK-docking sites have not been extensively studied. In our experience, FXFP sites do not bind nearly as well as D-sites, but we have not studied this rigorously.

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[illegible]

Fig. 1. Activation loop regions of selected MAPKs between conserved kinase subdomains VII and VIII. The fly, worm, yeast, dicty and plant sequences are from *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Dictyostelium discoideum* and *Arabidopsis thaliana*, respectively. Plants (and the unicellular slime mold *D. discoideum*) do not appear to have TGY-containing p38-like kinases, reducing the likelihood that anti-phosphop38 antibodies will cross react with plant stress-activated MAPKs.

Fig. 2.

Detection of yeast MAPK activation with anti-phospho-ERK1/2 antibodies. The phosphorylated forms of the yeast Kss1 and Fus3 MAP kinases are indicated. Strains of the indicated genotypes were treated (or not) for 15 min with mating pheromone (phm), protein extracts were prepared, and MAPK phosphorylation levels were determined by immunoblotting with the phosphorylation-state specific antibody described in the text. One hundred micrograms of protein is loaded per lane. '7Δ' indicates a *ste7Δ* strain from which the MEK for the mating pathway has been deleted, and 'ΔΔ' indicates a *kss1Δfus3Δ* strain lacking both MAPKs.

Protein	Docking Site	Reference
MEK1	Y192	[29]
MEK2	Y192	[29]
MKK3	Y192	[66]
MKK6	Y192	[66]
MKK4	Y192	[30]
MKK7	Y192	[31]
ySTE7	Y192	[25,47]
yDIG1	Y192	[24]
yDIG2	Y192	[24]
yFAR1	Y192	[59]
JIP1	Y192	[67]
JIP3	Y192	[68]
c-Jun	Y192	[49]
ATF2	Y192	[17]
ELK1	Y192	[48]
MKP1	Y192	[69]
MKP2	Y192	[70]
MKP3	Y192	[71]
PTP-SL	Y192	[72]
HePTP	Y192	[73]
MNK1	Y192	[55]
MNK2	Y192	[55]
RSK1	Y192	[74]
RSK2	Y192	[50]
yRck2	Y192	[75]
Elk1	Y192	[51]
c-fos	Y192	[76]
Fra1	Y192	[77]
Fra2	Y192	[77]
c-Myc	Y192	[77]
Vinexin	Y192	[78]
KSR	Y192	[51]
MKP1	Y192	[63]
yDig1	Y192	[24]

Fig. 3.

Selected MAPK docking sites from human and yeast MAPK-interacting proteins. Yeast proteins start with a 'y'. References are as follows: (D-site class) MEK1, MEK2 [29]; MKK3, MKK6 [66]; MKK4 [30]; MKK7 [31]; ySTE7 [25,47]; yDIG1, yDIG2 [24]; yFAR1 [59]; JIP1 [67]; JIP3 [68]; c-Jun [49]; ATF2 [17]; ELK1 [48]; MKP1 [69]; MKP2 [70]; MKP3 [71]; PTP-SL [72]; HePTP [73]; (MAPKAPK class) MNK1, MNK2 [55]; RSK1 [74]; RSK2 [50]; yRck2 [75]; (DEF-site class) Elk1 [51]; c-fos [76]; Fra1, Fra2, c-Myc [77]; Vinexin [78]; KSR [51]; MKP1 [63]; yDig1 [24].

Fig. 4. Docking interaction between a MAP kinase and one of its substrates. The small substrate contains a MAPK-docking site and two phosphoacceptor target sites that are phosphorylated by the MAPK.

Table 1

Sample yield calculations from *in vitro* translation reactions^a

A	B	C	D	E	F	G	H
Protein	%incorp. [³⁵ S]met	[met] in lysate fm/μL (= nM)	(B × C) [met] incorp. into protein (nM)	# met's in protein	(D/E) [Protein] (nM)	Protein mass kDa (=ng/μm)	(F × G/1000) (ng/μl)
c-Fos	0.134	10,000	1340	9	149	41	6.1
c-Jun	0.126	12,500	1575	10	158	36	5.7
Fus3	0.092	6,250	575	19	30	37	1.1
Hog1	0.093	10,000	930	10	93	45	4.2
Kss1	0.100	10,000	1000	15	67	41	2.7
Ste7	0.061	10,000	610	7	87	65	5.7

^a Column B is the percent incorporation of [³⁵S]methionine into protein. C is the concentration of endogenous methionine in the lysate. D is the calculated concentration of methionine incorporated into protein. E is the number of methionines in the protein coding sequence. F is the calculated concentration of the *in vitro*-translated protein in the reaction. G is the protein mass in kilodaltons, and H is the calculated yield of the *in vitro*-translated protein expressed as weight protein per volume lysate.

Table 2

Binding assay data for the interaction between MKK4 and JNK3^a

Experiment	³⁵ S-labeled protein (5 nM)	GST-fusion	μg	μM	% binding ^b	K _d (μM) ^c
A-1	MKK4	GST-JNK3	40	4.0	5.4	70.0
A-2	MKK4	GST-JNK3	40	4.0	8.6	42.6
A-3	MKK4	GST-JNK3	40	4.0	16.9	19.7
A-4	MKK4	GST-JNK3	40	4.0	12.1	29.1
A-5	MKK4	GST-JNK3	40	4.0	9.7	37.1
A-6	MKK4	GST-JNK3	10	1.0	3.6	27.0
A-7	MKK4	GST-JNK3	10	1.0	5.0	19.0
Mean						34.9
Standard deviation						17.7
Standard error						7.2
B-1	JNK3	GST-MKK4 ₁₋₉₄	50	6.9	12.6	48.3
B-2	JNK3	GST-MKK4 ₁₋₉₄	50	6.9	39.1	10.8
B-3	JNK3	GST-MKK4 ₁₋₉₄	50	6.9	15.2	38.9
B-4	JNK3	GST-MKK4 ₁₋₉₄	40	5.6	12.3	39.4
B-5	JNK3	GST-MKK4 ₁₋₉₄	10	1.4	7.3	17.6
B-6	JNK3	GST-MKK4 ₁₋₉₄	10	1.4	10.5	11.8
B-7	JNK3	GST-MKK4 ₁₋₉₄	10	1.4	8.6	14.8
B-8	JNK3	GST-MKK4 ₁₋₉₄	10	1.4	6.7	19.2
Mean						25.1
Standard deviation						14.7
Standard error						5.6

^aData are taken from Ho et al. [30]. Binding reactions (200 μL) contained ~1 pmole (~5 nM) ³⁵S-labeled, *in vitro*-translated protein and 10–50 μg (1.0–6.9 μM) GST fusion protein.
^bPercent of the input ³⁵S-labeled protein which bound to the GST fusion protein.
^cCalculated based on the known input concentrations and percent binding.