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MEK Partner 1 (MP1): Regulation of Oligomerization in MAP Kinase Signaling

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Abstract Specificity in signal transduction can be achieved through scaffolds, anchors, and adapters that assemble generic signal transduction components in specific combinations and locations. MEK Partner-1 (MP1) was identified as a potential ''scaffold'' protein for the mammalian extracellular signal-regulated kinase (ERK) pathway. To gain insight into the interactions of MP1 with the ERK pathway, we analyzed the ability of MP1 to bind to MEK1, ERK1, and to itself, and the regulation of these interactions. Gel filtration of cell lysates revealed two major MP1 peaks: a broad high molecular weight peak and a 28 kDa complex. An MP1 mutant that lost MEK1 binding no longer enhanced RasV12-stimulated ERK1 activity, and functioned as a dominant negative, consistent with the concept that MP1 function depends on facilitating these oligomerizations. Activation of the ERK pathway by serum or by RasV12 did not detectably affect MP1–MP1 dimerization or MP1–MEK1 interactions, but caused the dissociation of the MP1-ERK1 complex. Surprisingly, pharmacological inhibition of ERK activation did not restore the complex, suggesting that regulation of complex formation occurs independently of ERK phosphorylation. These results support the concept that MP1 functions as a regulator of MAP kinase signaling by binding to MEK1 and regulating its association with a larger signaling complex that may sequentially service multiple molecules of ERK. J. Cell. Biochem. 94: 708–719, 2005.

Key words: ERK1; MAP kinase; MEK1; MP1; scaffold

The extracellular signal-regulated kinase (ERK) cascade is one of the several MAP kinase cascades that display conservation of protein sequence and a three-kinase architecture [Cobb and Goldsmith, 1995; Lewis et al., 1998], but which are activated in response to different physiologic signals. The ubiquitous activation of ERKs raises important questions of how specificity of signaling is achieved. Scaffolding of upstream activators and downstream targets, appropriate for the cell type and prevailing extra-cellular condition, is one mechanism by which the efficiency and specificity of signaling can be regulated. This has been clearly demonstrated in *S. cerevisiae* [Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994; Herskowitz, 1995; Posas and Saito, 1997], and similar interactions have been reported for several mammalian proteins. JNK activator MKK4 organizes a mitogen-activated protein kinase (MAPK) module consisting of MEKK1, MKK4, and JNK1 [Xia et al., 1998], and JNK-interacting protein 1 (JIP1) operates in the JNK pathway and selectively binds to JNK, the JNK

Abbreviations used: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HA, hemagglutinin; KSR, kinase suppressor of Ras; LPA, lysophosphatidic acid; MEK, MAP kinase/ERK kinase; MP1, MEK partner 1; PMA, phorbol myristate acetate.

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activator MKK7, and the MKK7 activators mixed-lineage kinase 3 (MLK3) and dual leucine zipper-bearing kinase (DLK) [Whitmarsh and Davis, 1998; Whitmarsh et al., 1998]. In the ERK pathway, kinase suppressor of Ras1 (KSR1) serves as a scaffold by interacting with the protein kinases c-Raf, MAP kinase/ERK kinase (MEK), and ERK [Michaud et al., 1997; Xing et al., 1997; Denouel-Galy et al., 1998; Yu et al., 1998], forming high molecular weight complexes [Stewart et al., 1999; Nguyen et al., 2002] and, in addition, KSR1 is required for efficient ERK activation [Nguyen et al., 2002; Lozano et al., 2003].

The existence of a scaffold protein for the mammalian ERK kinase pathway was predicted on the basis that activated mutants of MEK1, deleted for a proline rich sequence (PRS) between subdomains IX and X, failed to transform fibroblasts [Catling et al., 1995] and activate endogenous ERK1 [Dang et al., 1998]. MEK partner 1, or MP1, was identified in a yeast two-hybrid screen with MEK1 as bait [Schaeffer et al., 1998]. MP1 specifically binds MEK1 and ERK1, but not the closely related proteins MEK2 or ERK2. Binding specificity is dependent on the MEK1 PRS. In vitro, MP1 enhances the activation of MEK1 by Raf, and when overexpressed in cells, MP1 can selectively enhance the activation of ERK1 and activation of a reporter driven by the transcription factor Elk1.

While it has been proposed that MP1 functions as an adapter or scaffold to enhance the efficiency of the MAP kinase cascade, little is known about its regulation or mechanism of action. Moreover, its small size (13.5 kDa) raises questions as to whether its cellular function is analogous to STE5 and other scaffolds or adapters. We performed deletion mutagenesis of short sequences of MP1 to understand how its interactions with other proteins could modulate the ERK pathway. An MP1 mutant in which amino acids 62–73 were deleted, dimerized efficiently but did not associate with MEK1 or enhance RasV12-stimulated ERK activity. Activation of the ERK pathway by serum and RasV12 caused the dissociation of the MP1–ERK1 complex. The MP1–MEK1 interaction appeared not to be regulated by signaling through this pathway. These results support the concept that MP1 functions as a regulator of MAPK signaling by binding to MEK1 and regulating its association with a larger signaling complex that may sequentially service multiple molecules of ERK.

MATERIALS AND METHODS

Cell Culture, Transfections, and Plasmids

CCL39 and HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. All transfections were performed using LipofectAMINE (Gibco BRL). FLAG-tagged wt-MP1 [Schaeffer et al., 1998] and HA-MEK1 [Catling et al., 1995] constructs have been described previously. Dr. Channing Der provided HA-tagged RasV12 and HA-tagged ERK1. Myc-tagged MP1 was a gift from Lukas Huber.

Antibodies and Other Reagents

Antibodies were obtained as follows: rabbit antibody against MP1 was raised against a synthetic peptide corresponding to amino acids 2–15; p-MAP kinase antibody from Calbiochem, La Jolla, CA; MEK1/2 antibody from Cell Signaling, Beverly, MA. Anti-ERK1 blotting in gel filtration fractions were performed using a monoclonal antibody (Upstate Biotechnology, Lake Placid, N.Y.) Anti-FLAG M2 monoclonal antibody from Sigma, St. Louis, Missouri; PD098059 was obtained from Calbiochem.

CoImmunoprecipitations

Twenty-four hours post-transfection cells were harvested in FLAG-lysis buffer [50 mM tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM EDTA, and 0.5 mM EGTA (pH 7.3) supplemented with 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.2 mM Na_3VO_4, and protease inhibitors] and immunoprecipitations were performed as described [Schaeffer et al., 1998]. To see regulation of MP1–ERK1 and MP1–MP1, 24 h after transfection the cells were starved for 5 h and either left untreated or stimulated for 10 min with 10% FBS. For regulation studies of MP1–ERK1 by Ras V12, parallel cultures were treated with 50 μm PD098059 for 5 h.

Kinase Assays

CCL39 cells were transfected with as described above. Following day cells were harvested in FLAG-lysis buffer and the ERK kinase activity was determined in HA–ERK1 immune complexes (HA, hemagglutinin) as described previously [Jelinek et al., 1994]. Myelin basic

protein (MBP) was cut from the membrane and radioactivity quantified by Cerenkov counting. HA–ERK1 was visualized by Western blotting using 12CA5 antibody.

Gel-Filtration

Clarified whole cell lysate of 250 μl (containing ~1–2 mg of protein) was loaded on a 16 × 240 mm Superose 12 gel filtration column (Pharmacia, Upsalla, Sweden) equilibrated in FLAG-lysis buffer without Triton X-100. The flow rate was 0.2 ml/min and 0.25 ml fractions were collected. The column was calibrated using molecular weight standards from Pharmacia: Mr 13,700 RNase, Mr 25,000 chymotrypsinogen, Mr 43,000 ovalbumin, Mr 67,000 albumin, Mr 158,000 aldolase, Mr 232,000 catalase, Mr 2,000,000 blue dextran 2000.

Metabolic Labeling and Phosphopeptide Mapping

Confluent cultures of CCL39 cells in 100 mm diameter plates were labeled in 5 ml of labeling medium [phosphate free RPMI 1640 (GibcoBRL) supplemented with pyruvate containing 1 mCi/ml of carrier-free ^{32}Pi for 3 h. Extract preparation and immunoprecipitations were performed as described above.

Isoelectric Focusing (IEF)

Isoelectric focusing was performed in 15 × 0.15 cm^2 acrylamide rods, using the gel composition as described earlier [Celis et al., 1992]. Carrier ampholines (BioRad, Hercules, CA) composition were 20% pH 5–7, 20% pH 7–9, and 60% pH 3.5–10. Immunoprecipitated FLAG–MP1 was eluted from FLAG resin using 0.1 mg/ml of FLAG peptide and applied per tube. The tubes were filled by overlaying the sample with a buffer containing 1% carrier ampholines (composition as described above) and 9M urea. Focusing was conducted for a total of 12 h using voltage stepping: 2 h at 200 V and 12 h at 800 V.

RESULTS

Protein Complexes Containing MP1

Because MP1 is small compared to other known scaffolds, we hypothesized that it interacted with multiple proteins to form large oligomeric complexes in cells, and that these interactions were essential for signaling function. To examine the range of MP1 interactions with endogenous cellular proteins, lysates of CCL39 fibroblasts were fractionated by gel filtration FPLC (Superose 12), the fractions were separated by SDS polycrylamide gel electrophoresis and probed by Western blotting for the mobility of MP1, ERK, and MEK. MP1 was found predominantly in two regions: a broad peak spanning fractions 6–22 (Fig. 1A, left panel) and a lower molecular weight complex (LMWC) of ~28 kDa in fractions 30–34 (Fig. 1A, right panel). MEKs and ERKs were identified in fractions expected for the monomeric proteins (F26 to F28 for MEK; F28 to F30 for ERK) but also cofractionated with MP1 in fractions 20–22 (>200 kDa) consistent with the occurrence of ternary complexes. Only a minor portion of MEK was detected in the highest molecular weight regions, but both ERKs and a substantial portion of the MP1 were detected even in the void volume. The presence of MP1 in fraction 6 (void volume) to fraction 22 shows that MP1 is part of a large complex that may involve other proteins besides MEK1 and ERK1. The presence of MP1 in large complexes is consistent with its engagement in multiple protein–protein interactions as expected of a component of a scaffolding complex.

The presence of MP1 in the ~28 kDa fraction suggested that MP1 could form dimers. MP1 has previously been shown to heterodimerize with the endosomal protein p14 [Wunderlich et al., 2001; Teis et al., 2002], which would yield a 28 kDa peak. To determine whether MP1 might also form homodimers, expression constructs encoding FLAG-tagged MP1 or Myc$_6$–MP1 were transfected into CCL39 cells and FLAG–MP1 was immunoprecipitated. The coimmunoprecipitation of Myc-tagged MP1 was then assessed by gel electrophoresis and western blotting. As is evident from Figure 1B, MP1 with six Myc tags that shifts MP1 mobility from ~15 to ~35 kDa coprecipitated with FLAG–MP1, implying the existence of MP1 multimers. Moreover, coimmunoprecipitation of untagged MP1 with FLAG–MP1 could also be detected, although the amount of untagged MP1 in these coimmunoprecipitates was modest (Fig. 1C).

The recently determined crystal structure of MP1 predicts that formation of MP1–MP1 homodimers would be structurally unfavorable due to steric incompatibilities between a loop formed from residues 62–70 [Lunin et al., 2004]. We generated serial deletion mutants of MP1

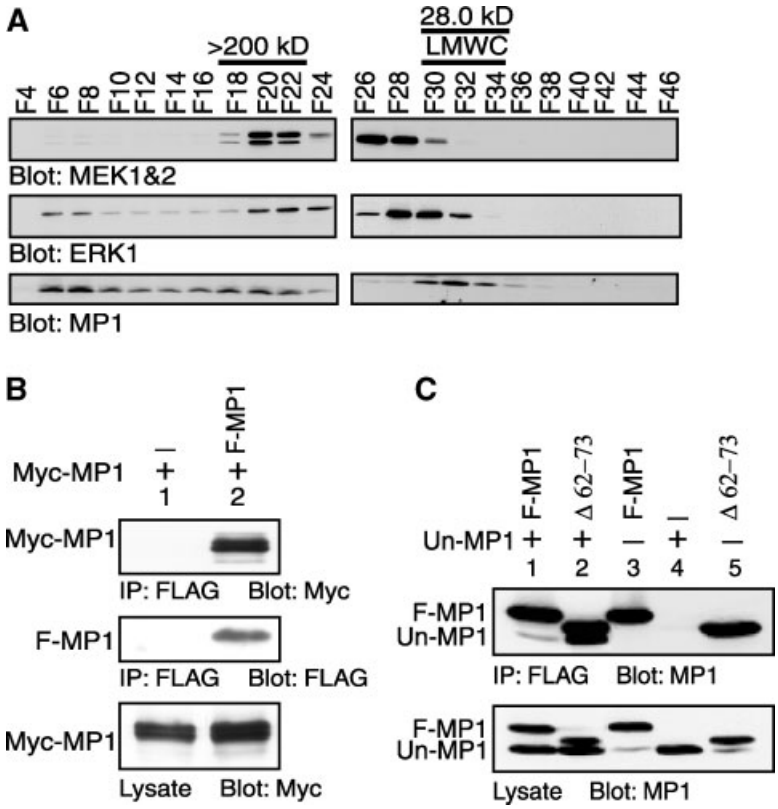

Fig. 1. Protein complexes containing MEK Partner-1 (MP1). **A**: Whole cell lysate of asynchronously growing CCL39 fibroblasts was fractionated by gel filtration and immunoblotted with MP1, MEK1 & 2, and ERK1 antibodies (MEK, MAP kinase/ERK kinase and ERK, extracellular signal-regulated kinase) **B**: FLAG-tagged wt-MP1 along with Myc_6-tagged wt-MP1 were transfected into CCL39 cells. FLAG–MP1 was immunoprecipitated using anti-FLAG antibody. Immunoprecipitated FLAG–MP1 was detected with MP1 antibody, Myc–MP1 was detected with Myc antibody. **C**: Either FLAG-tagged wt-MP1 or FLAG-tagged MP1Δ62–73 were transfected along with untagged wt-MP1 into CCL39 cells. FLAG–MP1 was immunoprecipitated using anti-FLAG antibody. Immunoprecipitated MP1 and expression levels of MP1 were detected with MP1 antibody. LMWC, low molecular weight complex; F-MP1, FLAG-tagged wild-type MP1; Un-MP1, untagged wild-type-MP1; Myc-MP1, Myc_6-tagged wild-type MP1.

(see below) and tested their ability to form MP–MP1 dimers. Deletion of amino acids 62–73 resulted in a significant increase in MP1 homo-oligomerization (Fig. 1C, see also Fig. 4D), consistent with the structural data.

Taken together, our data show that MP1 is capable of forming oligomers with multiple proteins, consistent with its putative role as a scaffold for MAPK signaling.

ERK1-MP1 Association Is Inhibited by RasV12 and Serum

To test whether MP1 hetero-oligomerization is regulated in coordination with MAPK pathway activation, we performed MP1–ERK1 coimmunoprecipitations with or without co-overexpression of oncogenic RasV12. Interestingly, the amount of ERK1 that coprecipitated with MP1 was dramatically reduced either by coexpression of RasV12 or when the cells were stimulated with FBS (Fig. 2A).

Since activation of the MAPK pathway resulted both in phosphorylation of ERKs and dissolution of the MP1–ERK1 complex, it seemed possible that phosphorylation of ERK would result in disruption of the MP1–ERK1 complex. Consistent with this hypothesis, phospho-ERK was undetectable in the MP1 immunoprecipitates (data not shown). However, the disruption of the MP1–ERK1 complex in response to RasV12 was not reversed by pretreating the cells with MEK inhibitor PD098059 (Fig. 2A, lane 3). The PD098059 was effective at inhibiting ERK activation by Ras V12, as judged by the reduction of the mobility shift in the ERK1 blot and very weak signal of phospho-ERK in the p-ERK blot. In addition, under conditions where only a small portion of ERK was activated as

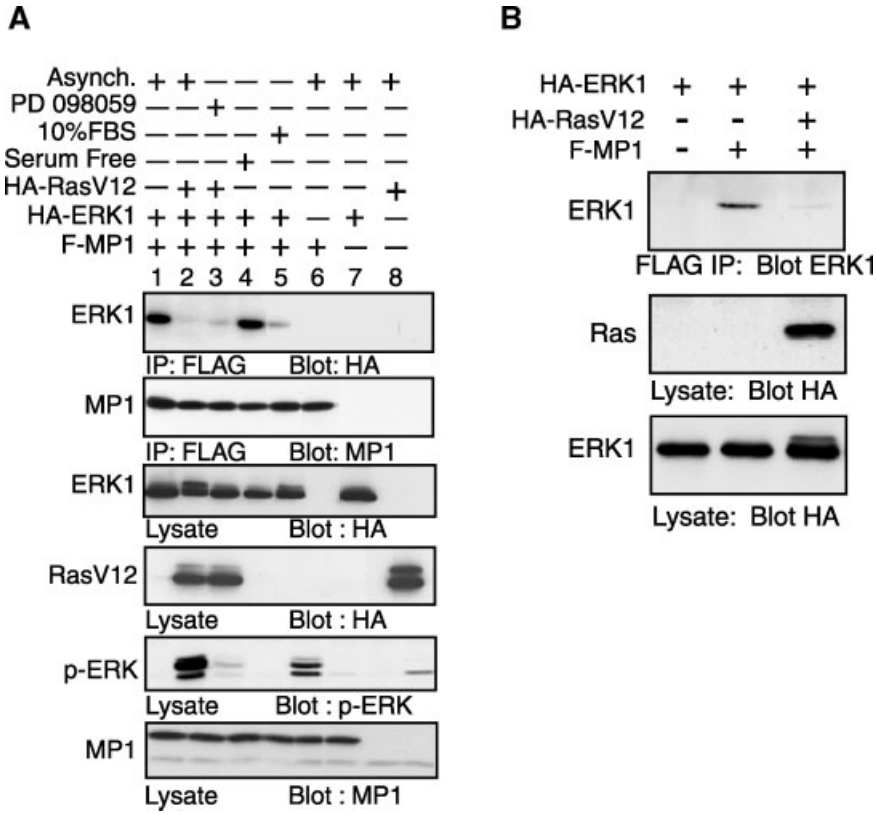

Fig. 2. Regulation of MP1–ERK1 interaction. **A**: CCL39 cells were cotransfected with FLAG–MP1, HA–ERK1, and HA–RasV12 or empty vector. The following day cells were serum starved for 5 h and either left untreated or stimulated with 10% serum for 10 min as indicated. In **lane 3**, cells were treated with 50 μm PD098059 to inhibit MEK activation. Cells used for **lanes 1**, **2**, and **6–8** were asynchronously growing cells. MP1 was immunoprecipitated using anti-FLAG antibody. Coimmunoprecipitated HA–ERK1 was detected using anti-HA antibody (HA, hemagglutinin). Lysates were also probed with HA and MP1antibodies to detect the expression of ERK1, RasV12, and MP1, respectively. Asynch., Asynchronously growing. **B**: CCL39 cells were cotransfected with FLAG–MP1, HA–ERK1, and HA–RasV12 or empty vector. MP1 immunoprecipitation and immunodetection were performed as described in A.

judged by mobility shift of ERK1, the MP1–ERK1 association was abolished (Fig. 2B). Thus, although disruption of the MP1–ERK1 complex correlated with ERK1 phosphorylation and activation, it seems likely that the regulation of this oligomerization occurs upstream of or parallel to ERK, and does not depend directly on ERK phosphorylation by MEK.

Similarly, we examined the regulation of MEK1–MP1 interactions in response to stimulation of the MAPK pathway (Fig. 3A). We performed MP1–MEK1 coimmunoprecipitations with or without co-overexpression of RasV12 and observed that MEK1 was associated with MP1 irrespective of the activation of this pathway.

We also tested whether MP1–MP1 interactions are subject to regulation. Since we found that deletion of amino acids 62–73 of MP1 results in a dramatic increase in MP1 homo-oligomerization (see below), MP1Δ62–73 was included in this experiment. We found that MP1–MP1 interaction was not decreased by Ras V12 or by serum (Fig. 3B).

MP1 Deletion Mutants

To define the region(s) of MP1 necessary for MEK1, ERK1, and MP1 binding, serial deletion mutants of 10–15 amino acids were generated throughout the protein (Fig. 4A) with a FLAG epitope at the N-terminus. The cDNAs encoding these deletion mutants were coexpressed with either HA–MEK1 or HA–ERK1 or MP1 and protein–protein associations determined by coimmunoprecipitation.

All the MP1 mutants were able to bind MEK1, with the exception of MP1Δ62–73, which failed to bind detectably (Fig. 4B, lane 7). MP1Δ14–25 (deletion at the N-terminus) and MP1Δ109–124 (at the C-terminus) exhibited enhanced binding

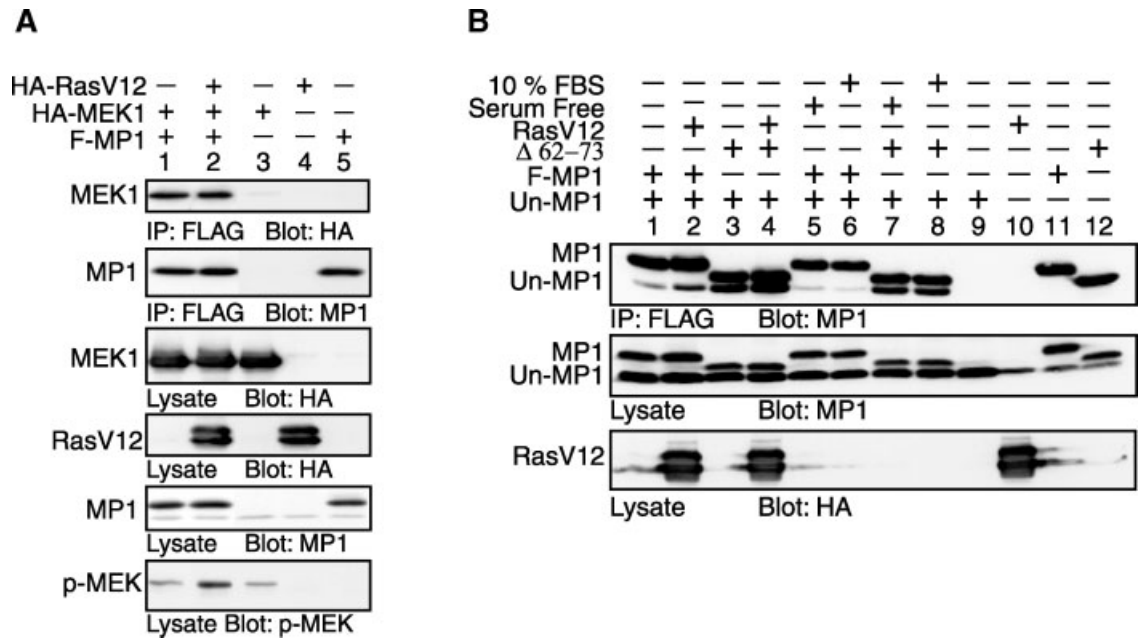

Fig. 3. Regulation of MP1–MEK1 and MP1–MP1 interactions. **A**: CCL39 cells were cotransfected with FLAG–MP1, HA–MEK1, and HA–RasV12 or empty vector. MP1 immunoprecipitation and HA–MEK1 detection were performed as described in Figure 2A. Lysates were also probed with HA-antibodies to detect the expression of MEK1 and RasV12. **B**: CCL39 cells were cotransfected with the indicated combinations of constructs. **Lanes 1–4** and **9–12** were asynchronously growing cells, **lanes 5–8** were serum starved and stimulated with 10% serum as indicated. Immunoprecipitation and immunodetection were performed as described in Figure 1C.

to MEK1 (Fig. 4B, lanes 3 and 11) at least as well as wt-MP1 (Fig. 4B, lane 1). Please note that the MP1 antibody was raised against the N-terminal amino acids and thus does not detect the Δ2–15 mutant.

We next determined the ability of MP1 mutants to bind ERK1. Several MP1 mutants displayed enhanced interactions with ERK1, including MP1Δ2–14, MP1Δ14–25, MP1Δ98–111, and MP1Δ109–124. MP1Δ62–73 bound to ERK1 to approximately the same extent as did wt-MP1 and this was in contrast to its inability to bind MEK1 detectably (Fig. 4C, lane 7). None of the MP1 mutants lost binding to ERK1 although MP1Δ26–37 association was very weak. Thus, there was no correlation, positive or negative, between interactions of the MP1 mutants with ERK1 versus MEK1.

Even though input DNA amounts were varied to achieve comparable expression of the various MP1 mutants, several mutants were expressed to low levels. Nevertheless, some of them exhibited robust binding to ERK1 (e.g., Fig. 4C, lanes 3, 10, and 11). To explain this surprising result, we considered the possibility that even the lower levels of MP1 were saturating for ERK1 binding. To test this concept, we varied the expression of MP1 wild type and the mutants MP1Δ14–25, MP1Δ62–73, and MP1Δ109–124 by varying the amounts of input DNA. We found that with the mutants and wild type, the amount of ERK1 coprecipitated varied directly with MP1 expression level (data not shown). Thus, we conclude that some of the MP1 mutants that express poorly also have an enhanced ability to coprecipitate ERK1. We do not have a mechanistic explanation for this finding.

Deletion of MP1 amino acids 62–73, which eliminates MEK1 binding, resulted in a dramatic increase in MP1 homo-oligomerization (Fig. 4D, lane 7). As MP1Δ62–73 does not bind MEK1 but exhibits strong homodimerization, we speculated that binding to MEK1 might compete with MP1 homodimerization. However, the increase of MEK1 expression by varying the amount of input DNA in transfections had no effect on the extent of MP1 dimerization (data not shown).

MP1Δ62–73 Does Not Enhance RasV12 Stimulated ERK1 Activity

As a putative scaffold, MP1 is predicted to enhance ERK1 activation by facilitating the

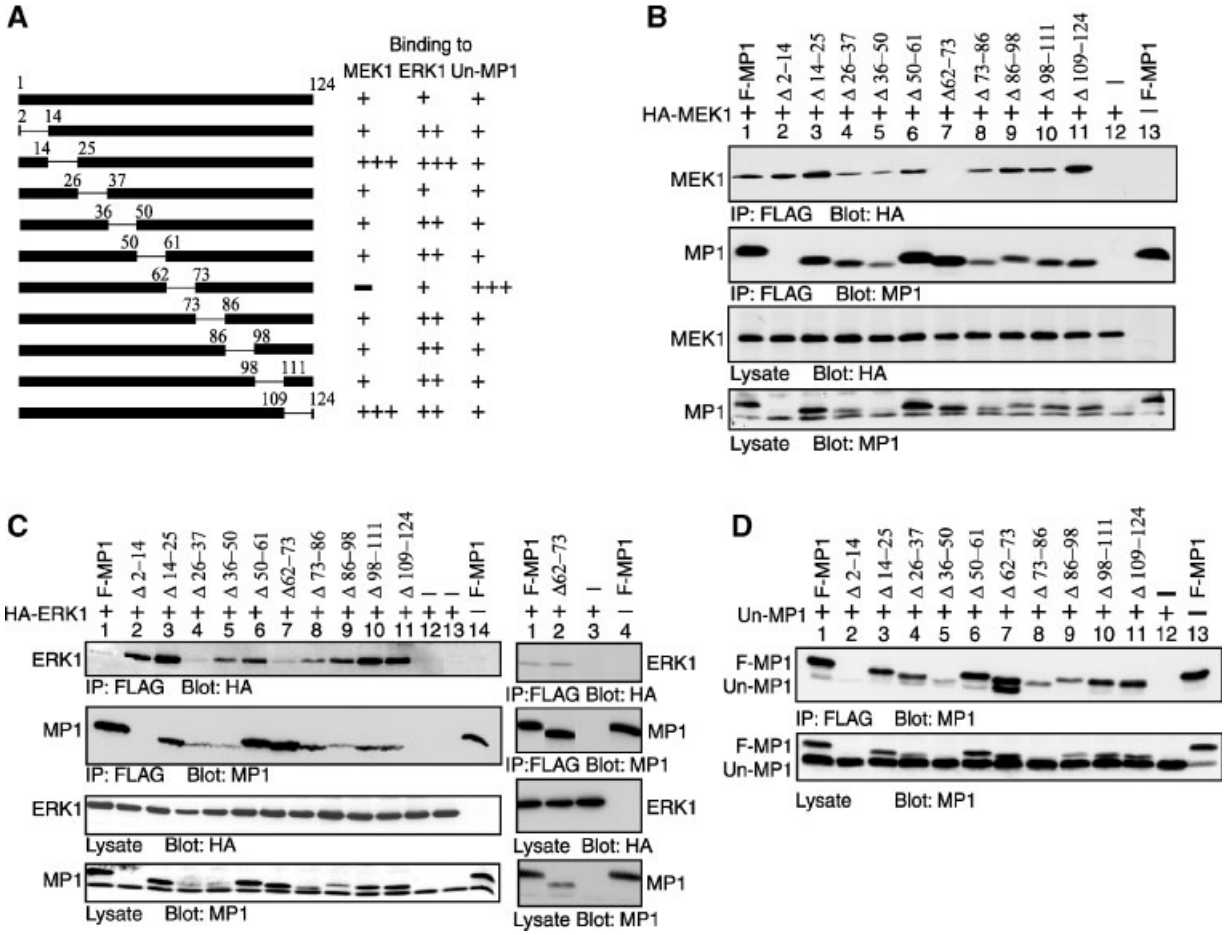

Fig. 4. Schematic representation of MP1 deletion mutants and interaction of MP1 mutants with MEK1 and ERK1. **A**: All MP1 mutants as well as wt-MP1 contained the FLAG-epitope tag at the N-terminus. Binding of MP1 mutants with MEK1, ERK1, and Untagged-MP1 (Un-MP1) is indicated as (+), lack of binding as (−). (++), and (+++) represents stronger binding. **B**: CCL39 cells were cotransfected with the indicated combinations of constructs. Immunoprecipitation and immunodetection were performed as described in Figure 3A. Low expression of certain MP1 mutants was consistently observed even at high input DNA concentration. MP1 antibody was raised against a peptide corresponding to amino acids 2–15, therefore in **lane 2**, this antibody failed to detect MP1. **C**: Binding of MP1 mutants with HA–ERK1 were checked as described above. **Right panel** compares binding of wt F-MP1 and Δ62–73 more precisely. **D**: Binding of MP1 mutants with untagged-wt-MP1 was assayed as described in Figure 1C. Expression level of MP1 was checked by blotting with MP1 antibody.

assembly of MEK1–ERK1 complexes. If this hypothesis is correct, we would predict that expression of MP1Δ62–73, which binds ERK1 but not MEK1, would be unable to enhance activation of the MAPK pathway and could even function as a dominant-negative. To test this, we transiently cotransfected CCL39 fibroblasts with HA-tagged ERK1, RasV12, and MP1Δ62–73 and ERK1 kinase activity was assessed in immune complex kinase assays. As expected, RasV12 stimulated ERK1 activity by a factor of 2–3. However, the transfection of MP1Δ62–73 resulted in significant dose-dependent inhibition of ERK activation and completely abolished ERK activity at the highest amount of MP1Δ62–73 (Fig. 5A). Although these data suggest that MP1Δ62–73 could act as an inhibitor of ERK signaling, the ability of scaffold proteins to stimulate signaling is critically dependent on the stoichiometry of the various components, and very high concentrations of scaffold can inhibit signaling by forming incomplete, nonproductive complexes. To test whether the ability of MP1Δ62–73 to inhibit signaling is a function of skewed stoichiometry, we directly compared the regulatory activity of wild-type MP1 with MP1Δ62–73 under conditions where each was expressed at the same

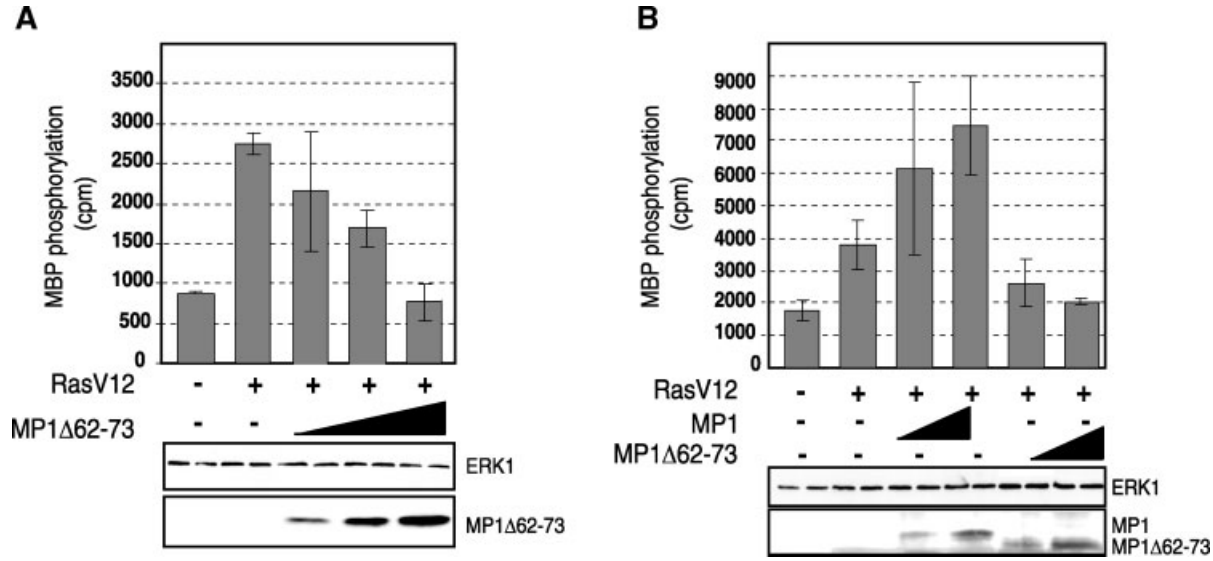

Fig. 5. FLAG–MP1Δ62–73 inhibits RasV12 stimulated ERK1 activity. **A**: CCL39 cells were transfected in duplicate with HA-tagged ERK1, HA-RasV12 and increasing amounts of FLAG–MP1Δ62–73. The following day the cells were serum deprived for 5 h, lysed and HA-tagged ERK1 was immunoprecipitated for an in vitro kinase assay using MBP as substrate. Immunoprecipitates were probed with ERK antibody to verify comparable levels of ERK proteins precipitated. To determine expression levels of FLAG–MP1Δ62–73, FLAG–MP1Δ62–73 was precipitated with FLAG antibody and immunodetected with MP1 antibody. **B**: CCL39 cells were transfected with HA-tagged ERK1, HA–RasV12, and either increasing FLAG–MP1 or FLAG–MP1Δ62–73. Kinase activity and ERK1 level were determined as described in A. To determine MP1 expression levels, lysates from duplicates were pooled and immunoblotted with MP1 antibody.

levels. As reported previously [Schaeffer et al., 1998], wt-MP1 enhanced ERK activity in a dose dependent manner (Fig. 5B). On the contrary, MP1Δ62–73 inhibited ERK activity even though the expression levels of wt-MP1 and MP1Δ62–73 were comparable. Thus, at the expression levels where wt-MP1 favors formation of productive signaling complexes and enhances ERK activation, MP1Δ62–73 forms nonfunctional complexes and inhibits signaling.

Phosphorylation of MP1

Since the association of MP1 and ERK1 is regulated by serum and Ras V12, which activate kinase cascades, we investigated whether MP1 gets phosphorylated. CCL39 cells, transiently expressing wt-MP1 or MP1Δ62–73, were labeled metabolically with ^{32}Pi as described in "Materials and Methods". Cultures were either left untreated or stimulated with 10% FBS for 15 min prior to lysis and MP1 was purified by immunoprecipitation. As is evident from Figure 6A, lane 4, a ^{32}P-labeled band of ~14 kDa was observed in the serum-treated sample. In addition, multiple higher molecular weight phosphoproteins reproducibly appeared in the MP1-expressing serum-stimulated immunoprecipitates, as would be expected based on the data showing MP1 in high molecular weight complexes (Fig. 1A). Based on the signal intensity of the coimmunoprecipitating bands and the fact that MP1 was overexpressed in this transient transfection paradigm, we surmise that the stoichiometry of phosphorylation on MP1 is very low. Although we did not detect MP1 phosphorylation in every experiment, this signal was never detected when vector alone (Fig. 6A, lane 1) or MP1 mutant MP1Δ62–73 were expressed (Fig. 6A, lane 2).

Although, the presence of MP1 in the ~14 kDa ^{32}Pi band was established by probing it with MP1 antibodies, it does not rule out the possibility that the radioactive label is in some other protein of the same size which coimmunoprecipitates with MP1. To address this issue, the band was excised from the gel and sequenced by mass spectrometry. Only MP1 but no other protein was identified (data not shown). The identity of this 14 kDa band was further established by two-dimensional gel electrophoresis. Figure 6B, upper panel shows the autoradiograph of the ^{32}Pi band and Figure 6B, lower panel shows MP1 expression as determined by probing with MP1 antibodies. The bands

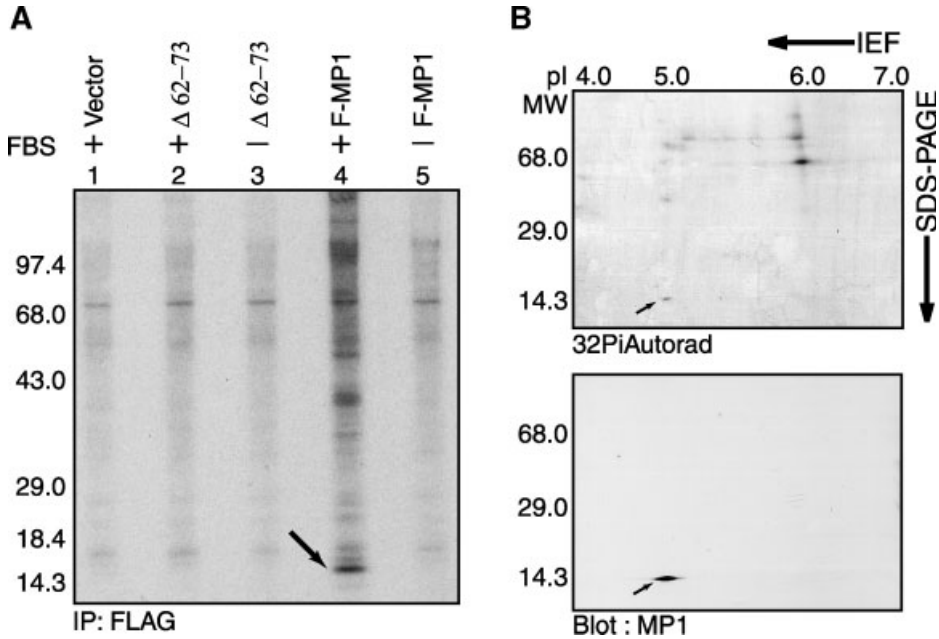

Fig. 6. Phosphorylation of MP1. **A**: CCL39 cells transfected with indicated constructs were labeled metabolically with 32-Pi as described in ''Materials and Methods''. MP1 was immunoprecipitated using anti-FLAG antibody, separated on PAGE and detected autoradiographically. FLAG–MP1 band is marked with an arrow. **B**: Two-dimensional electrophoretic analysis of metabolically labeled immunoprecipitated MP1 protein. The pH gradient of the first dimensional gel is indicated at the **top** of the figure. Molecular weight standards as run on SDS–PAGE in second dimension are indicated in the **left** margin. **Upper panel** shows the ^{32}Pi autoradiogram and **lower panel** shows blotting with MP1 antibody.

marked with an arrow in the upper panel and lower panel overlap each other.

A two-dimensional phosphopeptide map of FLAG-tagged wt-MP1 shows a single peptide and phospho amino acid analysis shows phosphorylation only on serine (data not shown).

DISCUSSION

MP1 Is Part of a Larger Oligomeric Protein Complex

Although a ternary complex of MP1–MEK1–ERK1 has previously been hypothesized, based on regulatory properties [Schaeffer et al., 1998], the structural and regulatory requirements of the MP1–MEK1 and MP1–ERK1 interactions were unknown. Gel filtration of whole cell lysates revealed a peak of MP1 corresponding to MP1 dimers, whereas the rest of the MP1 was distributed broadly in complexes greater than 200 kDa. The minimal size of the high molecular weight complexes is greater than what would be expected if this complex consisted only of MP1, ERK1, and MEK1 even if all the components were in dimers. Thus it is probable that other components of a signaling complex are present in these oligomers.

The presence of MP1 in a high molecular weight complex is consistent with its proposed role as a component of a signaling scaffold [Schaeffer et al., 1998; Schaeffer and Weber, 1999]. Presumably other members of the "signalosome" such as Raf and other scaffolds will also be found in these complexes. Although MP1 has not been found to interact with Raf in the two-hybrid system, weak interaction detected by coimmunoprecipitation (data not shown) is consistent with this concept. We have recently identified an MP1-binding protein by yeast two-hybrid analysis [Vomastek et al., 2004], and this protein coimmunoprecipitates with Raf and could thus serve a "bridging" function between the upstream and downstream components of the signaling module. A recent publication also demonstrates the presence of MEK and ERK in high molecular weight complexes and suggests that KSR can play a scaffolding function as well [Nguyen et al., 2002]. In addition, MP1 has been shown to interact with p14, a protein found in the late endosomal compartment [Wunderlich et al., 2001; Teis et al., 2002], and to interact with PAK1 (A. D. Catling, personal communication), a kinase important in signaling through integrins to the MAPK pathway [Frost et al.,

1997; Slack-Davis et al., 2003]. Recently, Lunin et al. [2004] determined the crystal structure of MP1 in complex with p14 and observed that these proteins have multiple protein folds, consistent with multiple simultaneous protein–protein interactions. The existence of multiple proteins which serve scaffolding, adapter, and/or coactivator functions would provide enhanced regulatory flexibility in the MAPK cascade, allowing these enzymes to couple specifically with diverse activators and effectors.

Although MP1 is specific for MEK1 and ERK1 [Schaeffer et al., 1998], and its recently discovered partner MORG1 is specific for enhancing ERK activation in response to serum, LPA, and PMA, but not EGF, we have not found MP1 to display agonist specificity: siRNA knockdown of MP1 had comparable effects on ERK activation whether serum, PMA, or EGF were used as agonists.

Presumably, one function of MP1 is to facilitate the activation and functioning of MEK1 by linking it to its signaling partners. The Δ62–73 MP1 mutant failed to bind to MEK1, did not enhance activation of ERK1, and functioned as a dominant negative, consistent with this concept.

It is clear that, in addition to high molecular weight signaling complexes, MP1 is also capable of forming dimers: a substantial portion of the MP1 was found to migrate at 28 kDa, the expected size of a dimer. It is uncertain what percentage of these dimers are heterodimers with p14 and other proteins, and what percentage are homodimers. Immunoprecipitation of FLAG-epitope-tagged MP1 coimmunoprecipitated the untagged or the Myc-tagged species, indicating that homodimerization is possible. However, only a small percentage of MP1 was detected in homodimers, and thus it appears that homodimerization is inefficient. The crystal structure of MP1 predicts that the residues 62–70 would sterically hinder homodimerization [Lunin et al., 2004]. The fact that the Δ62–73 MP1 mutant, which lacks this region, displayed increased dimerization, is consistent with the structural prediction. A small portion of MP1 is phosphorylated and the Δ62–73 MP1 mutant fails to display this phosphorylation. Although we have not been able to directly map the phosphorylation sites on MP1, an attractive hypothesis is that phosphorylation in this loop can regulate dimerization and other protein–protein associations. The functional importance of MP1 dimerization is uncertain. It is possible that MP1 functions as a dimer or that the dimers represent an inactive "storage" form of the protein.

Site-directed mutagenesis revealed that the sites of homodimerization and of interaction with MEK1 were distinct. Deletion of amino acids 62–73, while causing enhanced dimerization, ablated MEK1 binding. Consistent with this, we have found that MEK1 binding does not compete with MP1 dimerization when cotransfected into cells at various levels of input DNA (data not shown).

The Δ62–73 mutant, while not binding MEK1, still bound ERK1. This shows that MEK1 is unlikely to serve as a bridging molecule between MP1 and ERK1, a theoretical possibility based on the observation that the N-terminus of MEK can interact with ERK [Bardwell et al., 1996, 2001; Fukuda et al., 1997; Tanoue et al., 2000].

MP1 Oligomerization Is Physiologically Regulated

The Ras/Raf/MEK/ERK pathway is controlled in large part by physical interactions between its component proteins [Kolch, 2000], and these in turn are regulated by signaling. Activation of Ras results in the binding of Ras to Raf, phosphorylation of Raf and MEK1 by PAK regulates the Raf–MEK1 and MEK1–ERK interactions [Frost et al., 1997; Slack-Davis et al., 2003]. Activation of ERK is associated with the release of ERK from its interaction with MEK. Similarly, we found that ERK is released from its interaction with MP1 following stimulation of signaling with serum or activated Ras V12. This is consistent with a role for MP1 as a MEK1 partner in the activation of multiple molecules of ERK1. The interaction of MP1 and ERK was not restored by inhibiting MEK with PD98059, thus demonstrating that the regulation occurs independently of ERK phosphorylation.

We did not detect regulated decreases in MP1 association with MEK1 following activation of the ERK pathway. An attractive concept is that the MEK1–MP1 complex is stable and reutilized in activating multiple ERK molecules, which then move to sites of action. However, because only a small fraction of MEK gets activated in response to serum, it is possible that the interaction of this fraction with MP1 is regulated and that this regulation is not reflected in the behavior of the bulk pool of MEK.

In sum, we have shown that MP1 fulfills many of the criteria expected of a small protein that is part of a "scaffolding" complex involved in the regulation of MAPK signaling: it can be isolated as part of a high molecular weight complex, a mutant MP1 that does not interact with MEK1 no longer is able to enhance the activation of ERK1, and the interaction of MP1 with ERK1 is negatively regulated upon activation of the signaling pathway. This potentially allows the MP1–MEK1 complex to service sequentially a large number of ERK1 molecules, thus amplifying the signal and permitting the movement of ERK1 to its sites of action. Current study is aimed at identifying other components of the large signaling complexes and determining their functions.

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