Characterization of an ERK-binding Domain in Microphthalmia-associated Transcription Factor and Differential Inhibition of ERK2-mediated Substrate Phosphorylation*

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Efficient and specific signaling by mitogen-activated protein kinases (MAPKs) is enhanced by docking sites found on many MAPK substrates and regulators. Here we show that the MAPKs ERK1 and ERK2 form a stable complex ($K_d \sim 6 \mu M$) with their substrate the microphthalmia-associated transcription factor (MITF). Complex formation requires a domain of MITF of \( \sim 100 \) residues that is nearby, but C-terminal to, the MAPK phosphorylation site at Ser\(^{73} \). MITF derivatives lacking this ERK-binding domain do not bind ERK2 and are phosphorylated less efficiently by ERK2. The ERK-binding domain of MITF bears no obvious resemblance to previously characterized MAPK docking motifs; in particular, it does not contain a consensus D-site. Consistent with this, ERK2-MITF binding does not require the integrity of the CD/sevenmaker region of ERK2. Furthermore, D-site peptides, which are able to potently inhibit ERK2-mediated phosphorylation of the Elk-1 transcription factor ($IC_{50} = 3 \mu M$), are relatively poor inhibitors of ERK2-mediated phosphorylation of MITF, exhibiting >15-fold selectivity for inhibition of Elk-1 versus MITF. These observations demonstrate substrate-selective kinase inhibition: the possibility that small molecules that target docking interactions may be used to selectively inhibit the phosphorylation of a subset of the substrates of a kinase.

Signal transduction networks that regulate gene expression and metabolism are crucial for cell growth, differentiation, and survival. Accordingly, aspects of the pathology of many diseases result from defects in signaling pathways or the transcriptional responses they regulate (1).

Many extracellular signals are transmitted to downstream targets by an evolutionarily conserved protein kinase cascade designated the mitogen-activated protein kinase (MAPK)\(^2 \) cascade. The MAPK (also known as extracellular-signal-regulated kinase, or ERK) cascade is a three-kinase module that transmits signals from cell-surface receptors to downstream effectors (2). The MAPK is phosphorylated, and thereby activated, by upstream MAPK/ERK kinase (MEK), which has previously been activated by a MEK kinase, such as the proto-oncoprotein Raf. To further propagate the signal, activated MAPKs phosphorylate other kinases such as RSK1 (3), which then regulate downstream effectors. In addition, MAPKs directly phosphorylate many key effectors, including the ubiquitination of genes involved in differentiation, melanogenesis, and cell survival (9–11). MAPK haptinsufficient leads to Waardenburg syndrome type IIa, which is characterized by deafness and pigment defects caused by melanocyte loss in the inner ear and the skin (12). In addition, dominant-negative mutations in MITF are associated with another auditory-pigmentary disorder, Teitz syndrome (13, 14) (reviewed in Ref. 15). MITF is also a potential target in the treatment of skin cancer, because it may impart a selective advantage to melanoma cells at low expression levels (16, 17), while having an antiproliferative effect at higher expression levels (18).

During the differentiation of neural crest-derived melanocytes, activation of the c-Kit receptor tyrosine kinase by its ligand Steel factor initiates signaling through the Ras\(\rightarrow\)Raf\(\rightarrow\)MEK1\(\rightarrow\)ERK1/2 cascade, resulting in the phosphorylation of MITF, and the consequent induction of genes involved in differentiation (e.g. p21\(^{CIP1} \) (19)), melanogenesis (e.g. tyrosinase (20)), and melanocyte survival (e.g. Bcl2 (16)). ERK2 directly phosphorylates MITF on Ser\(^{73} \) (21); MITF is also phosphorylated at Ser\(^{409} \) by ERK-activated RSK1 (22). The dually phosphorylated MITF displays increased affinity for the transcriptional co-activator CREB-binding protein (23). Dually phosphorylated MITF is also targeted for ubiquitination and subsequent degradation, ensuring transient gene induction (22).

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 (24, 25). These docking sites are small regions within substrates, removed from the site of phosphorylation, that bind with relatively high affinity to MAPKs, in some cases showing specificity for particular MAPKs.

One class of MAPK-docking site, designated the “D-site,” has the consensus ($K/R)_1 X_1 X_2 X_3 \ldots X_9 \rightarrow \phi X_\delta$, where $\phi$ 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 (26–29), and were subsequently found in numerous MAPK-interacting proteins, including multiple MEKs, substrates, phosphatases, and scaffold proteins (reviewed in Refs. 24 and 25). Recent 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/seven- maker” region (30–32) and the “ED region” (33), whereas the hydrophobic submotif of the D-site binds to a nearby “hydrophobic docking groove” (30, 34, 35). A second docking motif, related to the D-site, is found in MAPK-activated kinases such as RSK1 (36). This motif (consensus L-X1,2-R/K-X1,3) also makes contacts with the CD/sevenmaker and ED regions (32, 33).

Another docking motif for MAPKs (consensus FXFP) has been named the “DEF motif” (37, 38). This motif binds to a hydrophobic pocket (distinct from the one that the D-site binds to) that is located below the MAPK active site. This pocket is occluded in unphosphorylated ERK2 (35); thus, the DEF motif preferentially binds to the dually phosphorylated, activated isoform of ERK2 (35).

Protein kinases are important therapeutic targets (39–42). Most existing kinase inhibitors bind to the active site, and thus inhibit the ability of a kinase to phosphorylate all of its substrates. The concept of kinase-substrate docking raises new possibilities for drug discovery (43–46). First, molecules that target docking interactions can also be effective inhibitors, as exemplified by the ability of D-site peptides to inhibit MAPK-dependent reactions in vitro (47–51) and in vivo (52, 53). Second, if MAPKs and other protein kinases use different parts of their surfaces to dock to different substrates, then it might be possible to selectively inhibit a subset of interactions, while leaving others relatively unimpaired. This could be advantageous in limiting toxicity and other side effects.

Here we report our characterization of an interaction between ERK1 and ERK2 and their substrate MITF and demonstrate that D-site peptides are selective for the inhibition of D-site-containing substrates, and do not effectively inhibit ERK2 phosphorylation of MITF in vitro.

**EXPERIMENTAL PROCEDURES**

*Plasmids for in Vitro Transcription/Translation*—The following mammalian genes were used in this study: human MITF (transcript variant 4, GenBank™/EBI accession number NM_002248), human MEK1 (MAP2K1, accession number NM_002755), MEK2 (MAP2K2, NM_030662), MKK6 (MAP2K6, NM_027588), and p38α (MAPK14, L35253), and rat ERK2 (M64300). Construction of pGEM4-Z MEK2 (48), pGEM4-Z-MKK6 (49), pGEM4-Z-ERK2 (48), and pGEM4-Z-p38α (47) are described in the citations given. To construct pGEM4-Z-MITF, the MITF coding region was amplified by high fidelity PCR using Pfu turbo DNA polymerase, primers Mi 1-X (5’-GCGAATTCCCAT-GTGGGAATGCGTAGAATA) and Mi X-419 (GCCGTTGACCTA-AAGTGTGCTCCGTCT), and a cDNA clone (National Institutes of Health, Image, ID no. 268834) obtained from the I.M.A.G.E. consortium (55) as the template. The PCR product was digested with EcoRI and Sall and inserted into the corresponding sites of pGEM4-Z (Promega). To construct many truncation mutants of pGEM4-Z-MITF, the MITF coding region was amplified using appropriate primers and pGEM4-Z-MITF as the template. The PCR products were digested with EcoRI and Sall and inserted into the corresponding sites of pGEM4-Z. Generation of pGEM4-Z-MITFΔ98–190, pGEM4-Z-ERK2–7m1, and pGEM4-Z-ERK2–7m2 was accomplished by site directed mutagenesis using the QuickChange kit (Stratagene) and confirmed by sequencing.

*Plasmids for the Production of GST Fusion Proteins*—The vector used for generating the GST fusion proteins was pGEXLB (48), a derivative of pGEX-4T-1 (Amersham Biosciences). Construction of pGEX-ERK1, pGEXLB-ERK2, pGEXLB-MEK2-(1–64), and pGEX-STE7-(1–198) has been described previously (27, 48). To construct pGEXLB-MITF-(35–198) and pGEXLB-MITF-(35–98), the coding regions were excised from pGEM4-Z-MITF-(35–198) and pGEM4-Z-MITF-(35–98), respectively, by digestion with EcoRI and Sall, and inserted into the corresponding sites of pGEXLB.

*Plasmids for Transfection into Mammalian Cell Lines*—To construct pcDNA-MITF-V5, the full-length MITF coding sequence was amplified by high fidelity PCR (using the cDNA Image clone as template), digested with EcoRI and Apal and ligated into the corresponding sites of the pcDNA3.1-V5 vector (Invitrogen). Similarly, pcDNA-MITF-(98–198)-V5 was constructed by amping the appropriate region of MITF, and ligating the resulting PCR product into the EcoRI and Apal sites of pcDNA3.1-V5. Plasmid pcDNA-MITF–ΔEBD-V5 (98–190) was constructed by site directed mutagenesis of pcDNA3.1-V5-MITF using primers that consisted of the DNA sequence of the regions that flank the DNA resulting in residues 98–190. The construction of pcDNA3.1-FLAG-ERK2 has been described previously (56).

*Binding Assays*—In vitro transcription and translation were performed using a coupled reaction (SP3, Novagen). The translation products were partially purified by ammonium sulfate precipitation as described previously (48). GST fusion proteins were expressed in bacteria, purified, and quantified as previously described (48, 57). The methods used in the co-sedimentation experiments, and the calculation of dissociation constants, have also been described previously (26, 48).

*Protein Kinase Assays*—The protein kinase assays for ERK2 phosphorylation of Elk-1 was performed as described previously (49). Kinase reactions (20 μl) for ERK2 phosphorylation of MITF contained kinase-assay buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol), 50 μM ATP, 1 μCi of [γ³2-P]ATP, 1 μM MITF (a fusion protein consisting of residues 35–198 or 35–98), and 10 units (~1 ng) of active mouse ERK2 (New England Biolabs). Reactions were performed for 20 min at 30 °C. MITF phosphorylation was quantified by SDS-PAGE followed by analysis of relative incorporation using a Phosphorimager (Amersham Biosciences).

*Proteins and Antisera*—GST-Elk-1 (307–428) and active mouse ERK2 were purchased from New England Biolabs, Inc. The anti-ERK antibody was from Santa Cruz Biotechnology. Mouse monoclonal anti-V5 antibody was obtained from Invitrogen. AlexaFluor-488-conjugated anti-mouse antibody was purchased from Molecular Probes, Inc.

*Co-sedimentation of ERK from HEK293 Cell Lysates*—HEK293 cells (obtained from ATCC) were cultured at 37 °C in a humid atmosphere containing 5% CO₂ in air. Cells (5 × 10⁵ per well) were seeded into 6-well dishes and grown to confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine. Twenty hours prior to harvesting, cells were transferred to media containing 0.5% serum. For epidermal growth factor stimulations, cells were incubated with 100 ng/ml epidermal growth factor (in phosphate-buffered saline) for 5 min prior to harvesting. Cells were then scraped into 0.5 ml of lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol) containing protease and phosphatase inhibitor cocktails (Sigma) (58). Lysates were centrifuged at 20,000 × g for 5 min to remove any insoluble material, and the resultant supernatants were pre-cleared with 20 μl of blocked 50% reduced glutathione beads for 30 min at 4 °C. Lysates were then incubated with the specified amounts of bead-bound GST fusion protein at 4 °C for 1 h. Beads were then washed twice with 0.5 ml of ice-cold cell wash buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and resuspended in SDSPAGE loading buffer. Samples were analyzed by Western blotting with anti-total-MAPK or anti-phospho-MAPK antibodies (Cell Signaling). Blots were then incubated with AlexaFluor-680/800 nm secondary antibody conjugates (Molecular Probes).
and were analyzed using the Odyssey Infra-Red Imaging System and software (LI-COR BioSciences) according to the manufacturer’s instructions.

Co-immunoprecipitation—HEK293 cells (5 × 10⁵) were seeded into 6-well culture dishes and were co-transfected with 4 µg of plasmid DNA encoding either V5-tagged full-length MITF (pcDNA3.1-V5-MITF), MITF-ΔEBD (pcDNA3.1-V5-MITF-ΔEBD), MITF-EBD (pcDNA3.1-V5-MITF-EBD), or empty vector (pcDNA3.1-V5), and 1 µg of plasmid encoding ERK2 (pcDNA3.1-FLAG-ERK2) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours post-transfection cells from two 35-mm wells were lysed into 200 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.2 mM NaVO₄, 50 mM glycerophosphate, protease inhibitor mixture (Sigma), pH 7.6) and centrifuged at 14,000 × g for 15 min at 4 °C. The supernatant lysates were then cleared with 20 µl of a 50% slurry of Protein G Plus/Protein A-agarose beads (Calbiochem) for 30 min at 4 °C. The cleared lysates were then incubated for 1 h at 4 °C with 20 µl of beads (50% slurry) that had been preincubated with 1 µl of anti-V5 antibody (30 min at 4 °C). The beads were washed twice with 0.5 ml of 20 mM Tris-HCl (pH 7.4), 1 M NaCl, once with 0.5 ml of 10 mM HEPES, 10 mM MgCl₂, and were finally resuspended in 1× SDS sample buffer. Samples were separated on standard SDS-polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride membranes. Primary antibody incubations were carried out overnight at room temperature, followed by 1-h incubations with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using an ECL detection kit (Pierce) according to the manufacturer’s instructions.

Immunofluorescence Microscopy—The protocol for immunostaining has been described previously (56).

RESULTS

MITF Binds ERK2 Specifically and with High Affinity—To assess the nature of the interaction between human ERK2 and its target transcription factor MITF, rat ERK2 was fused at its N terminus to Schistosoma japonicum glutathione S-transferase (GST), and the resulting fusion protein was expressed in bacteria and purified by adsorption to glutathione-Sepharose beads. ERK2 prepared in this manner is obtained in its unphosphorylated, unactivated state. GST-ERK2 (or GST alone as a negative control) was then incubated with full-length human MITF that had been produced in radiolabeled form by in vitro translation (Fig. 1). As a positive control, GST-ERK2 beads were incubated with radiolabeled human MEK2; we have previously shown that ERK2-MEK2 bind specifically, because precipitation of MITF protein did not occur when GST was used instead of the GST-ERK2 fusion protein. MEK2 also bound specifically to GST-ERK2, as previously reported (48). Additionally, full-length MITF also bound to a GST-ERK1, although with somewhat reduced affinity compared with the GST-ERK2 (data not shown; see also Fig. 3).

N-terminal 198 Residues of MITF Mediate Binding to ERK2—The first 198 residues of MITF contain the ERK2 phosphorylation site (Ser⁷³), as well as a transcriptional activation domain that shares a sequence and structural homology to the E1a transcriptional activation domain (23). Residues 199-288 contain the basic-helix-loop-helix leucine zipper (bHLH-LZ) DNA-binding domain, characteristic of the bHLH-LZ family of transcription factors (59, 60). A second transcriptional activation domain is contained within MITF residues 324–369 (61) (Fig. 2A). To localize the ERK-binding domain of MITF, polypeptide fragments of MITF were produced and tested for binding to GST-ERK2 (Fig. 2B). As shown in Fig. 2C, MITF₁⁻³⁵ and MITF₁⁻⁹⁻⁹⁻⁴⁻¹⁻¹⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻acerbased on the known input concentrations and percent binding for at least six different experiments (see Ref. 47 for details).

TABLE ONE: Select Kᵣ values

| Select Kᵣ values | ³⁵S-protein | GST fusion | Kᵣ(µM) | S.D.  |
|-------------------|-------------|------------|--------|-------|
| MITF              | GST-ERK2    | 5.7        | 1.3    |
| MITF(-1⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-|
inhibitory element that lowers affinity of MITF for ERK2. Second, MITF<sub>75–419</sub> bound to ERK2 with lower affinity than did either MITF<sub>70–419</sub> or wild-type MITF. Hence, the phosphoacceptor sequence, 71PNSP74, apparently contributed to the binding energy of the ERK2-MITF interaction, either by contacting unphosphorylated ERK2 directly, or by affecting the conformation of the ERK-binding domain. Finally, MITF<sub>98–419</sub> still bound ERK2, whereas MITF<sub>125–419</sub> did not. Thus, the N-terminal border of the ERK-binding domain is contained between residues 98–125.

The above results suggested that residues 98–198 of MITF contain the ERK-binding domain. In confirmation of this suggestion, MITF<sub>98–198</sub> bound to GST-ERK2 (Fig. 3A). Furthermore, an MITF derivative lacking residues 98–198 displayed markedly reduced binding to ERK2, whereas a derivative lacking the first 198 residues did not bind to ERK2 at all (Fig. 3B and C).

Typical MAPK docking sites can be constituted within short peptides (31, 47, 49). To determine if the ERK2-binding portion of MITF could be further localized to a short, contiguous, “peptide-like” region, a series of overlapping 15-mer peptides, covering the region between MITF residues 61 and 205, were synthesized and anchored to a cellulose membrane (62). This array was then incubated with radiolabeled ERK2. In contrast to MEK12–16 and MEK22–16 D-site peptides included, as controls, on the same array, none of the MITF-derived peptides bound appreciably to ERK2 (data not shown). Hence, there is no short contiguous stretch of amino acids within MITF residues 61–210 that is capable of binding to ERK2 with reasonable affinity and stability. This result, combined with the localization results described above, support the notion that the ERK-binding domain (EBD) of MITF is indeed perhaps best conceptualized as a globular domain contained within residues 98–198.
**MITF ERK-binding Domain**

The lack of interaction between ERK2 and MITF-ΔEBD was not due to mislocalization of MITF-ΔEBD, because both wild-type MITF and MITF-ΔEBD localized to the nucleus (Fig. 4C). This is consistent with the proposal that MITF residues 210–222 contain its primary nuclear localization signal (63).

To determine if the ERK-binding domain of MITF was sufficient to associate with ERK2 in cells, a plasmid expressing V5-tagged MITF<sub>98–198</sub> was co-transfected into HEK293 cells with the vector over-expressing ERK2, cells extracts were prepared, and MITF<sub>98–198</sub> was immunoprecipitated. As shown in Fig. 4D, ERK2 specifically co-precipitated with MITF<sub>98–198</sub>. In summary, as determined by co-immunoprecipitation, ERK2 and MITF stably associated in human cells, and the ERK-binding domain of MITF was necessary and sufficient for this interaction.

**GST-MITF Fusion Proteins**—To further assess the ERK-MITF interaction, we attempted to express several GST-MITF fusion proteins. We were unable to obtain soluble GST-MITF derivatives containing MITF residues 1–34; however, fusion proteins lacking these residues were soluble (data not shown). It is interesting to note that residues 1–34 of MITF also inhibited binding to ERK2 (see above), although this inhibition must have been due to a different mechanism (i.e. not because of reduced solubility), because all the in vitro-translated proteins were comparably soluble. Soluble fusion proteins that could be purified in high yield were obtained by fusing residues 35–198 of MITF to GST, yielding GST-MITF<sub>35–198</sub>; this protein contains both the MAPK-phosphorylation site and the ERK-binding domain of MITF. As a control, GST-MITF<sub>35–98</sub>, which contains the MAPK phosphorylation site but lacks the ERK-binding domain, was also created. These proteins were then purified from bacteria and used in several assays, described below.

**N Terminal of MITF Selectively Binds to ERK2**—To explore the specificity of the MITF-MAPK interaction, three proteins that were not expected to bind to MITF were produced by in vitro translation and tested for binding to GST-MITF<sub>35–198</sub>. These proteins were human MAPK/ERK kinases MEK1 and MKK6 and the bacterial enzyme β-galactosidase. Also included in this analysis were ERK2 and the human p38α MAPK. As is shown in Fig. 5, none of the in vitro-translated proteins bound to either GST or GST-MITF<sub>35–98</sub>, and neither MEK1, MKK6, nor β-galactosidase bound appreciably to GST-MITF<sub>35–198</sub> as expected. Furthermore, when compared with ERK2, p38 bound to GST-MITF<sub>35–198</sub> relatively poorly (Fig. 5). Thus, the N terminus of MITF binds to ERK2 (and ERK1) with specificity.

Fus3 and Kss1, two yeast MAPK proteins, also bound with relatively high affinity to GST-MITF<sub>35–198</sub> but not to GST-MITF<sub>35–98</sub> or GST (data not shown). These two proteins are presumed orthologs of ERK1/2 (64) and have been shown to have binding specificities similar to ERK1/2 (31, 48).

**Both Phosphorylated and Unphosphorylated ERK Bind to MITF**—To examine the binding of phosphorylated ERK1 and ERK2 to MITF, the following experiment was performed (Fig. 6A): human HEK293 cells were serum-starved for 16–20 h to shut down flux through the Ras/MAPK pathway. Half of the cells were then stimulated for 5 min with epidermal growth factor to activate ERK1 and ERK2. Cell lysates were prepared, co-sedimented with GST-MITF fusion proteins, and analyzed by immunoblotting with an anti-total-ERK antibody or anti-phospho-ERK antibodies. A fusion of GST to the N terminus of human MEK2 was also included in this experiment as a positive control; this protein has been previously shown to bind with high affinity to ERK2 (47). As shown in Fig. 6B (top left panel), unphosphorylated ERK2 bound to GST-MEK2<sub>1,64</sub> and GST-MITF<sub>35–198</sub>, but not to GST or GST-MITF<sub>35–98</sub> unphosphorylated ERK1 was difficult to visualize in

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**Picture Descriptions**

**Figure 4.** ERK2 and MITF co-immunoprecipitate from HEK293 cells, and the ERK-binding domain of MITF is necessary and sufficient for this interaction. A, V5-epitope-tagged MITF derivatives were expressed in HEK293 cells and immunoprecipitated; co-precipitation of ERK2 was assessed. B, HEK293 cells were co-transfected with plasmid DNA encoding V5-tagged MITF<sub>98–198</sub> or empty vector, and plasmid encoding ERK2. C, Immunochemical signals (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm. D, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD (a derivative lacking residues 98–198), or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm. E, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD, or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm. **Figure 5.** A, Western blotting of proteins expressed in HEK293 cells and their interaction with mitogen-activated protein kinase (MAPK) family members.; B, immunofluorescence microscopy using an anti-V5 antibody. Cells were also counterstained with 4’,6-diamidino-2-phenylindole to visualize nuclei. Scale bar = 20 µm. C, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD, or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm. D, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD, or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm.

**Figure 6.** A, Western blotting of proteins expressed in HEK293 cells and their interaction with mitogen-activated protein kinase (MAPK) family members.; B, immunofluorescence microscopy using an anti-V5 antibody. Cells were also counterstained with 4’,6-diamidino-2-phenylindole to visualize nuclei. Scale bar = 20 µm. C, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD, or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm. D, HEK293 cells were co-transfected with plasmid DNA encoding either V5-tagged full-length MITF, MITF-ΔEBD, or empty vector. Cells were lysed 48 h post-transfection, and MITF was immunoprecipitated using an anti-V5 antibody. Immunoprecipitated complexes (IP) along with 10% of the input lysate (10%) were analyzed by Western blotting using antibodies against the V5 tag and ERK2. Both MITF and MITF-ΔEBD run as doublets. The asterisk indicates a cross-reacting band present in the input (10%) lane. The western blot was further stained with DAPI to visualize nuclei. Scale bar = 20 µm.
MITF ERK-binding Domain

FIGURE 5. MITF 98–198 preferentially binds to ERK2. A, ERK2 and other proteins were tested individually for binding to GST-MITF35–198. B, 35S-labeled ERK2, p38, MEK1, M KK6, or β-galactosidase were prepared by in vitro translation and partially purified by ammonium sulfate precipitation, and portions (5% of that used in the binding reaction) were resolved on a 10% SDS-polyacrylamide gel (lane 1). Samples (~1 pmol) of the same proteins were incubated with ~200 μg of GST, GST-MITF35–198, or GST-MITF35–98 bound to glutathione-Sepharose beads, and the resulting bead-bound complexes were isolated by sedimentation and resolved by 10% SDS-PAGE on the same gel. The gel was analyzed by staining with Coomassie Blue (not shown). Also shown are lysate samples corresponding to 5% of the total protein input (lanes 1 and 6). Equal sedimentation of the GST fusions was verified by staining with Coomassie Blue (not shown).

this experiment. Furthermore (lower right panel), both active ERK1 and active ERK2 also bound to GST-MEK21–64 and GST-MITF35–198, but not to GST or GST-MITF35–98. Hence the ERK-binding domain of MITF can bind to both phosphorylated and unphosphorylated ERK1 and ERK2 in cell extracts.

Mutations in the CD/7m Domain of ERK2 Have No Effect on MITF Binding—Examination of residues 70–198 of MITF did not reveal the presence of any known MAPK docking site motifs. In particular, there was neither a clear consensus D-site ((K/R)1–3-X1–7-φXβφ), nor a consensus DEF-site (FXFP). Using the program Scansite (65), one weak match to the D-site consensus182KRELTAICIFP191, was found. Alteration of this putative D-site by site-directed mutagenesis, however, did not diminish MITF-ERK binding (data not shown), indicating that this region is not a functional D-site mediating the ERK-MITF interaction. Scansite also identified a weak match to the DEF-site in the same region, 186CIFP191. Interestingly, residues 187–192 (ACIFPT) are encoded by an exon that is spliced out of one of the two common isoforms of MITF (66, 67). Deletion of residues 187–192 (mimicking the alternative splice, and both removing the putative DEF-site and truncating the putative D-site) did not affect the binding of MITF1–198 to GST-ERK2 (data not shown). Thus, neither of the putative MAPK docking sites in the ERK-binding domain of MITF are required for MITF-ERK binding.

D-site-mediated interactions (as well as those mediated by the related MAPK-docking site found in MAPKAPKs) require the integrity of an acidic patch on the surface of the MAPKs designated the “conserved docking” (CD) region (32). The CD region is also called the sevenmaker region, after the allele of the Droso phila MAPK7 rolled that first indicated this region’s importance in MAPK signaling (68). The fact that the ERK-binding domain of MITF did not contain a consensus D-site, nor more than two acidic residues in a row, suggested that it did not bind to the CD/sevenmaker region. To directly test this suggestion, the ability of ERK2 derivatives with mutations in the CD region to bind to MITF was assessed. Two ERK2 mutants were tested. The ERK2–7m1 mutant (D319N) is equivalent to the sevenmaker mutation in rolled (D334N). The ERK2–7m2 double mutant (D316A, D319A) is equivalent to mutations in yeast Kss1 and Fus3 that caused a severe defect in binding to Ste7MEK. As well as to the D-site-containing substrates Dig1 and Dig2 (31).

In vitro-translated and radiolabeled ERK2, ERK2–7m1, and ERK2–7m2 were incubated with GST-MITF35–198, GST-MITF35–98, or GST. In addition, GST fusions to two MEK proteins, yeast Ste7 (GST-Ste71–172) and human MEK2 (GST-MEK21–64), were also included. As shown in Fig. 7, wild-type ERK2 bound to GST-Ste7 and GST-MEK2, as previously reported (47, 48). In contrast, the CD/sevenmaker region mutants of ERK2 were defective in binding to GST-Ste7 and GST-MEK2; this was expected, based upon previously reported findings with similar MAPK mutants (31, 32, 69). Notably, both the
CD/sevenmaker-region mutants of ERK2 bound to MITF_{35–198} just as well as did wild-type ERK2. Finally, none of the ERK2 derivatives bound to MITF_{35–98} or GST. Thus, the ERK2 CD/sevenmaker mutations abolished binding to D-site containing MEKs, but did not affect binding to MITF. From this it can be concluded that the integrity of the CD/sevenmaker region is not required for the ERK2-MITF interaction.

The MITF-ERK2 Interaction Potentiates ERK2 Phosphorylation of MITF—The docking of MAPKs to substrates containing D-sites or FXFP sites has been shown to facilitate the MAPK-dependent phosphorylation of those substrates (29, 37, 70). To ascertain whether the ERK-binding domain of MITF potentiated the phosphorylation of MITF by ERK2, in vitro kinase assays were performed using activated ERK2 as the enzyme, and GST-MITF_{35–198} or GST-MITF_{35–98} as the substrate. As shown in Fig. 8A, GST-MITF_{35–198}, which lacks the ERK-binding domain, was phosphorylated 2- to 4-fold less efficiently than GST-MITF_{35–198}, which contains the ERK-binding domain. MITF_{35–198}S73A, in which the phosphoacceptor serine 73 was changed to an alanine, was not phosphorylated at all, as expected (Fig. 8B and C).

D-site Peptides Differentially Inhibit ERK2 Phosphorylation of Elk-1 versus MITF—D-site-based peptides have been shown to inhibit the phosphorylation of D-site-containing substrate proteins by MAPKs (47, 49, 71). Most of this inhibition is presumably caused by the peptide binding to the MAPK and competitively inhibiting the docking of the MAPK to its substrate (49). The results described above indicated that the ERK-binding domain MITF does not contain a D-site, and that the MITF-ERK2 interaction does not involve the MAPK CD/sevenmaker region, one of the patches on the MAPK surface that D-sites bind to. Thus we hypothesized that the ERK2-mediated phosphorylation of MITF might not be strongly inhibited by D-sites peptides (Fig. 9A). To test this idea, in vitro kinase assays were performed using activated...
ERK2 as the enzyme and GST-Elk1_{407-428}, GST-MITF_{35-198}, or GST-MITF_{35-98} as the substrate. To certain reactions, the Ste7 D-site peptide was added to a final concentration of 5–50 μM. This peptide (sequence FQRKTLQRNKLGLNLHPD) was chosen because it displayed the most potent inhibitory activity of several that were tested previously (48, 49). A peptide consisting of a scrambled version of this sequence (scram7, sequence RLQPNLKDLHNHLGR) was added to certain reactions as a control. The results are shown in Fig. 9 (B and C).

The Elk-1 transcription factor contains one of the first characterized D-sites for ERK2 (29). Accordingly, ERK2-mediated phosphorylation of GST-Elk was potently inhibited by the native Ste7 D-site (IC_{50} \approx 3 μM), but not by the scrambled peptide, as we have previously reported (49). In contrast, the phosphorylation of GST-MITF_{35-198}, which does not contain a D-site, was inhibited much less efficiently by the Ste7 peptide site (IC_{50} \approx 50 μM). Furthermore, this weak inhibition was also seen with MITF_{35-98}, which lacks the ERK-binding domain (EBD); thus, it was apparently not caused by the peptide blocking the productive interaction of the MITF EBD with ERK2, and may have instead been caused by a peptide-induced conformational change in ERK2 (30, 34).

The selectivity of the Ste7 D-site for inhibiting ERK2-mediated phosphorylation of Elk-1 versus MITF was estimated by dividing the IC_{50} for its inhibition of ERK2 phosphorylation of MITF (50 μM) by the IC_{50} for its inhibition of ERK2 phosphorylation of Elk-1 (3 μM). This calculation indicated a selectivity of >15-fold. Thus, peptides that block D-site-mediated docking interactions can be used to selectively inhibit the phosphorylation of a D-site containing substrate (Elk-1), while not substantially impairing the phosphorylation of a different substrate that does not contain a D-site (MITF).

**DISCUSSION**

MAPKs, and many other kinases, bind to their substrates and regulators using high affinity docking interactions as part of a bipartite recognition strategy (docking site and phosphorylation site) that enhances signal transmission and may enable a "double selection" for fidelity (24, 25, 72). In this study, we have demonstrated a stable, high affinity interaction between the MAPKs ERK1 and ERK2 and their transcription factor target MITF. Both phosphorylated and unphosphorylated ERK1 and ERK2 were found to bind to MITF with an affinity in the low micromolar range, comparable to the affinities seen for other MAPK-interacting proteins (31, 47, 73) (Figs. 1 and 6). The high affinity ERK-binding region of MITF was localized to a domain of ~100 amino acids lying C-terminal to the ERK2 phosphorylation site at Ser^{73} (Figs. 2 and 3). This ERK-binding domain was necessary and sufficient for the stable association of ERK2 and MITF in human cells (Fig. 4). The ERK-binding domain exhibited specificity for MAPK binding, because it did not bind to irrelevant proteins such as MEK1, MKK6, and β-galactosidase (Fig. 5). The presence of the ERK-binding domain increased the efficiency of Ser^{73} phosphorylation (Fig. 8). Thus we conclude that, as it does with other many substrates, ERK2 uses a bipartite recognition strategy to facilitate the specific and efficient phosphorylation of MITF.

The ERK-binding domain is devoid of known MAPK-docking sites such at the D-site or the FXFP site. Consistent with the lack of a D-site or a related docking site containing a basic submotif, we found that ERK-MITF binding did not require the integrity of the MAPK CD/sevemmaker region, a patch of acidic residues required for MAPK binding to multiple substrates and regulators (31, 32) (Fig. 7). These results suggested that MITF might bind to a part of the MAPK surface different from those regions that are utilized for binding to D-sites (30, 32–35). Consistent with this idea, we showed that a D-site-based peptide that potently inhibited the ERK2-mediated phosphorylation of a D-site-containing substrate (Elk-1) only weakly inhibited the phosphorylation of MITF (Fig. 9). These results provide a proof-of-principal for the possibility of small-molecule MAPK inhibitors that would block the phosphorylation of some substrates while allowing phosphorylation of others (Fig. 9A).

**Diversity of MAPK-binding Motifs**—There are other examples of proteins that bind to MAPKs using domains that do not contain D-sites or FXFP sites. These include yeast Ste12, which binds preferentially to unphosphorylated Kss1MAPK (74); mammalian PEA-15, which binds to ERK1/2 via its DED domain (75); and Ets-1, which binds via its pointed domain (76). The ERK-binding domain of MITF is neither a pointed nor a DED domain and does not have any recognizable homology to Ste12; thus, it would appear to constitute a novel MAPK-binding domain. Of course, it is possible that MITF and these other proteins share overlapping binding surfaces on the MAPK. Interestingly, the ERK-binding domain contains within it the strongest of the two transcriptional activation domains of MITF (23).
Progress toward More Specific Kinase Inhibitors—There is increasing interest the possibility of developing drug candidates that modulate kinase function in a selective mechanism-based manner, rather than by competing for ATP binding (43, 45, 77). Clearly it might be useful to develop "substrate-selective kinase inhibitors": agents that could inhibit certain phosphorylation events while leaving others unperturbed. Some progress along these lines has been made recently. A peptide inhibitor of cyclin-dependent kinase-mediated Rb phosphorylation has been developed that does not affect cyclin-dependent kinase-mediated histone phosphorylation (78). CMPD1 is a small molecule inhibitor of p38 MAPK that exhibits substrate-selective inhibition of MK2 versus ATF-2 (79). In addition, the finding that the FXFP-site binds to a different part of the MAPK surface than the D-site (35) suggests that it should be possible to specifically inhibit FXFP interactions.

Here, we used a model peptide based on the D-site class of MAPK docking sites and showed that this peptide substantially inhibited ERK2-mediated phosphorylation of Elk-1, but did not dramatically affect the phosphorylation of MITF. In fact, the selectivity of the D-site peptide for inhibiting Elk-1 versus MITF phosphorylation was over 15-fold. The simplest explanation of these results is that the peptide bound to ERK2 and blocked the ERK2-Elk-1 docking interaction by competing with the D-site on Elk-1 but did not block the ERK2-MITF interaction. Thus, the D-site peptide acted as a substrate-selective ERK2 inhibitor.

A potential caveat to this interpretation lies in the fact that the D-site peptide did inhibit MITF phosphorylation somewhat, with an IC50 of ~50 µM. It seems unlikely that this effect was due to the peptide competing with the ERK-binding domain of MITF, because the peptide also inhibited the phosphorylation of an MITF fragment that lacked the ERK-binding domain, and did so with an IC50 of ~50 µM. One possible explanation for this inhibition is that the binding of the D-site peptide may have caused a conformational change in ERK2 that reduced its catalytic activity. Recent structural studies of MAPK-D-site complexes are consistent with this possibility (30, 34).

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