MEK1 Binds Directly to βArrestin1, Influencing Both Its Phosphorylation by ERK and the Timing of Its Isoprenaline-stimulated Internalization

Dong Meng1, Martin J. Lynch1, Elaine Huston1, Michael Beyermann2, Jenny Eichhorst1, David R. Adams2, Enno Klussmann2, Miles D. Houslay1, and George S. Baillie3

From 1Neuroscience and Molecular Pharmacology, Faculty of Biomedical and Life Sciences, Wolfson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom, the 2Leibniz-Institut für Molekulare Pharmakologie, Campus Berlin-Buch, Robert-Rössle-Strasse 10, 13125 Berlin, Germany, and the 3Department of Chemistry, Heriot-Watt University, Riccarton Campus, Edinburgh EH14 4AS, Scotland, United Kingdom

βArrestin is a multifunctional signal scaffold protein. Using SPOT immobilized peptide arrays, coupled with scanning ala-nine substitution and mutagenesis, we show that the MAPK kinase, MEK1, interacts directly with βarrestin1. Asp26 and Asp9 in the N-terminal domain of βarrestin1 are critical for its binding to MEK1, whereas Arg77 and Arg98 in the N-terminal domain of MEK1 are critical for its binding to βarrestin1. Wild-type FLAG-tagged βarrestin1 co-immunopurifies with MEK1 in HEK293 cells, whereas the D26A/D29A mutant does not. ERK-dependent phosphorylation at Ser412 was compromised in the D26A/D29A-βarrestin1 mutant. A cell-permeable, 25-mer N-stearoylated βarrestin1 peptide that encompassed the N-domain MEK1 binding site blocked βarrestin1/MEK1 association in HEK cells and recapitulated the altered phenotype seen with the D26A/D29A-βarrestin1 in compromising the ERK-dependent phosphorylation of βarrestin1. In addition, the MEK disruptor peptide promoted the ability of βarrestin1 to co-immunoprecipitate with endogenous c-Src and clathrin, facilitating the isoprenaline-stimulated internalization of the β2-adrenergic receptor.

The βarrestins are multifunctional signal scaffolding proteins that play a pivotal role in the desensitization process that regulates the functioning of many key heptahelical G protein-coupled receptors (GPCRs)1, 2. The β2-adrenergic receptor (β2-AR) has provided a critical functional paradigm in elucidating this fundamental process, where agonist occupancy triggers its phosphorylation by G-protein-coupled receptor kinase, thereby initiating the recruitment of cytosolic βarrestins (3, 4). By associating with agonist-occupied receptors, βarrestins attenuate GPCR functioning by both regulating interaction with signal-transducing G-proteins and facilitating GPCR internalization, leading to either recycling or degradation of the targeted receptor (5). Indeed, the interaction of receptor-recruited βarrestin with clathrin cages provides a key part of the paradigm for the deactivation of select ligand-bound GPCRs (6). βarrestins can also deliver sequestered cAMP phosphodiesterase-4 isoforms, particularly PDE4D5, to the site of cAMP synthesis associated with the β2-AR, thereby contributing a key part of the cellular desensitizing system for cAMP (5, 7, 8).

In the resting state, cytosolic βarrestin1 proteins are constitutively phosphorylated by extracellular signal-regulated kinase (ERK) at Ser412, located within their distal C terminus (9, 10). The agonist-stimulated recruitment of βarrestin to GPCRs, such as the β2-AR, leads to the dephosphorylation of βarrestin1 at this site. This event acts as a molecular switch, allowing for the internalization of the β2-AR-sequestered βarrestin complex. Thus, ERK-phosphorylated βarrestin1 is unable to associate with clathrin cages, whereas this constraint is removed upon its dephosphorylation (9, 11). Dephosphorylation of Ser412 is also thought to be a determinant for the association of βarrestin1 with c-Src and the phosphorylation of dynamin, a key feature in receptor internalization (12). Thus, the ERK2-dependent Ser412 phosphorylation and dephosphorylation of βarrestin1 provides a pivotal molecular switch that determines the association of βarrestin1 with the endocytic machinery governing internalization of the β2-AR (11).

The activation of ERK critically depends upon its phosphorylation by the MAPK kinase, MEK1. Compartmentalization and fidelity of this action is endowed by the ability of these proteins to interact and dock to each other, where a motif called the CD domain has been shown to play an underpinning role (13). In particular, MEK1 is known to bind to a negatively charged cluster that consists of two aspartate residues separated by any other two amino acids (DXXD) (14). The functioning of MEK1 has been shown to be integral to many cellular processes, such as transcription regulation, proliferation, and differentiation. Therapeutically, inhibitors of MEK1 have been developed as potential therapeutics for cancer and, more recently, have become the focus of development for treating chronic inflammatory disorders, such as rheumatoid arthritis.
and asthma (15–17). It has previously been suggested that MEK may form a complex with ERK and with β-arrestin (10, 18) that creates a “signalosome” capable of disseminating MAPK signals to defined intracellular compartments. In other studies, it has been suggested that constitutive β-arrestin-ERK1 complexes probably recruit MEK1 through its binding to ERK as a consequence of active Ras-dependent signaling (19–23). However, a recent report suggests that MEK can bind directly to β-arrestin1 (24) within sites on both N and C domains. Critically, however, the exact nature of MEK interaction with β-arrestin1 is unclear. Given that β-arrestin1 is ERK-phosphorylated in the cytosol (9) and the critical importance of this event to β-arrestin-mediated GPCR internalization, the nature of MEK1 interaction with β-arrestin1 warrants investigation. Here then, we evaluate MEK1 interaction with the signal scaffolding protein, β-arrestin1, demonstrating that MEK1 binds directly to β-arrestin1, allowing the identification of a functionally active peptide disruptor of this complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bradford reagent was from Bio-Rad. The following antibodies were used at concentrations of 1:2000: MEK1 (2352), MEK1/2 (4694), ERK2 (9108), ERK1/2 (4694), and β-arrestin1 phospho-Ser112 (2416) (Cell Signaling Technology, Beverly, MA); β2-AR (sc-569), glutathione S-transferase (GST) (sc-53090), clathrin HC (sc-12734), and hemaggutinin probe (sc-7372) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); β-arrestin1 phospho-Ser112 (44-200; lot 0102), anti-Src-pan (44-6555), and anti-His-horseradish peroxidase (P/N 56-0707) (Invitrogen); and FLAG-horseradish peroxidase (A8592) (Sigma). Monoclonal anti-sera specific for the PDE4D subfamily was a kind gift from Dr. S Wolda (ICOS Corp., Seattle, WA). Active GST-MEK1 (SGT-220) and inactive HisMEK1 (14-706) was a kind gift from Dr. S Wolda (ICOS Corp., Seattle, WA). Monoclonal antibodies specific for ERK and β-arrestin-ERK1 complexes were purchased from Millipore. HEKB2 cells were a kind gift from Prof. Enno Klusmann (Leibniz-Institut für Molekulare Pharmakologie, Berlin).

**Cell Culture and Drug Additions**—HEKB2 cells were seeded onto poly(L-lysine)-treated coverslips at 20% confluence. After treatment with indicated ligands, cells were fixed for 10 min in 4% (w/v) paraformaldehyde, pH 8.5, and dialyzed three times with ice-cold resuspension buffer. The fusion proteins were eluted by the addition of 5 mM glutathione, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and complete protease inhibitor mixture (Roche Applied Science) for at least 30 °C. Bacteria were harvested by centrifugation at 6,000 × g for 15 min at 4 °C, and the bacterial pellet was frozen at −80 °C overnight. The bacterial pellets were resuspended in 10 ml of ice-cold resuspension buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and complete protease inhibitor mixture) and sonicated with four 30-s bursts at the maximal setting. Triton X-100 was added to a final concentration of 0.02%, and cell debris was then removed by centrifugation at 15,000 × g for 10 min at 4 °C. The cleared supernatant was incubated with one-tenth volume of pre-equilibrated glutathione-Sepharose beads on an orbital shaker for 30 min at 4 °C. The beads were collected by centrifugation at 13,000 × g for 1 min and washed three times with ice-cold resuspension buffer. The fusion proteins were eluted by the addition of 5 mM glutathione, 50 mM Tris-HCl, pH 8.0, and dialyzed three times against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% glycerol. The purified fusion proteins were stored at −80 °C until required.

**Plasmid Construction and Site-directed Mutagenesis**—FLAG-tagged β-arrestin1, which was generated by an insertion of the full open reading frame of β-arrestin1 into the Not1 site of pcDNA3 (Invitrogen), was a kind gift from Dr. Graeme B. Bolger (University of Alabama at Birmingham). Primers 5′-GGGAAAAAGCGGGGCTTTTTGTGGCCCAACCATCGACCTCGTGGACCC-3 and 5′-GGTCCACAGGAGGTGATGTTGGGCCACAAAGGCCGCTTCCCC-3 were used to generate the full-length D26A/D29A-β-arrestin1 mutant with the QuikChange site-directed mutagenesis kit (Stratagene). The pCHA-MEK1 construct was a gift from Prof. Walter Kolch (Beattson Institute for Cancer Research, Glasgow, UK). Details of the construct were described previously (28). Primers 5′-GGAGCCTTGACGACGACGAGGGGGCCGCTCGAGGGGCTTTCGAGC-3 and 5′-GGTCCACAGGAGGTGATGTTGGGCCACAAAGGCCGCTTCCCC-3 were designed to generate R47A/K48A/R49A-MEK1.

**Microscopy**—HEKB2 cells were seeded onto poly(l-lysine)-treated coverslips at 20% confluence. After treatment with indicated ligands, cells were fixed for 10 min in 4% (w/v)
parafomaldehyde followed by three washes with Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.4). The coverslips were mounted to microscope slides with Immunomount. Cells were visualized using the Zeiss Pascal Laser-scanning confocal microscope (Zeiss, Oberkochken, Germany). Fluorescent peptide entry into cells was done using an LSM510 laser-scanning microscope (Zeiss).

In Vitro Pull-down Using Purified Proteins—1 nmol of purified GST or GST-β-arrestin1 was mixed with an equal amount of HisMEK1 (Millipore) in 0.5 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.5% Triton X-100). The mixture was incubated for 1 h at 4 °C, and then 50 μl of anti-polyhistidine-agarose beads (A5713; Sigma) were added in for an overnight incubation. Beads were collected by 10,000 × g centrifugation for 1 min and washed three times with binding buffer before loading to an SDS-polyacrylamide gel for protein separation.

Western Blotting and Protein Estimation—Immunoblotting was done as previously described (5), using 25–50 μg of cellular protein/well. After treatment, HEKβ2 cells or HEK cells were washed twice with PBS before being scraped into 3T3 lysis buffer (25 mM HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, pH 7.5) with added protease inhibitors (Complete Protease Inhibitor Mixture; Roche Applied Science). Proteins were separated by PAGE and transferred to nitrocellulose for Western blotting. Protein concentrations of cell lysates were determined isolated by incubation with protein A-coated Sepharose beads for 1 h before retrieval by refrigerated centrifugation at 10,000 × g, for 5 min. Target molecule-immunoglobulin conjugates attached to the beads were then washed in phosphate-buffered saline (PBS) three times.

Internalization of β2-AR Assay—Briefly, cells were grown to 80% confluence in Petri dishes and treated with 0.3 mg/ml disulfide-cleavable biotin (Pierce) in PBS at 4 °C for 30 min (5 ml/well). Cells were then washed with precooled PBS three times to cease biotinylation. The cells were then treated with the appropriate ligands, with or without peptides for the indicated times. Samples were washed with PBS two times, and the biotinylated receptors were stripped with stripping buffer (0.05 M glutathione, 0.3 M NaCl, 0.075 M NaOH, 1% FBS/newborn calf serum in PBS) at 4 °C for 30 min. Cell extracts were resuspended in radioimmunoprecipitation buffer (150 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, and 0.1% Triton X-100, pH 7.4), and biotinylated receptors were immunologically isolated using immobilized NeutrAvidin (Pierce) beads at 4 °C overnight. Samples were then washed three times with radioimmunoprecipitation buffer or PBS, and samples were analyzed via SDS-PAGE. Total receptor amounts were visualized and quantified using anti-β2-AR antiserum (sc-569; Santa Cruz Biotechnology).

Analysis by Microscopy—Slides were examined using a fluorescent imaging microscope at a magnification of ×43 and imaged for phase contrast; GFP fluorescence, and 4',6-diamino-
MEK1 Binds Directly to βArrestin1

midodo-2-phenylindole staining. Images of three random fields of view were taken from each slide, and all cells within these areas were quantified. After correction for background, areas were outlined to define the intracellular compartment of the cell, using phase contrast to define the plasma membrane, and the number of cells present in each field of view was quantified using 4',6-diamidino-2-phenylindole staining of the nuclei of the cells. The image analysis software, Metamorph 7.0, was used to find spots within the intracellular compartment of the cell, and the number of spots per cell was quantified and used as an indication of the internalization of the β2-adrenoreceptor. Student’s t test was used for statistical analysis.

RESULTS

Purified MEK1-GST Binds Directly to the βArrestin Peptide Array—MEK1 is a 45-kDa MAPK kinase that activates ERK in a classic amplification cascade. Previous studies (10) utilizing multiple transfection of key intermediates have indicated that MEK1 is able to interact with βarrestin. However, it is unclear from these reports as to whether this occurs directly or involves a bridging molecule, such as ERK, which is well known to bind to MEK and has been shown to associate directly with β-arrestin (19, 31, 32). Recently, evidence has emerged suggesting that MEK may bind to both the N and C domains of βarrestin1, although the exact nature of the binding sites remains to be determined (24).

In order to determine whether MEK1 has the potential to interact directly with βarrestin, we first employed peptide array analysis. This provides a novel and powerful technology for gaining insight into the basis of specific protein–protein interactions. Indeed, we have used this to considerable advantage to exhaustively map sites of interaction between both of the scaffolding proteins, βarrestin (27) and RACK1 (7), with the cAMP-hydrolyzing PDE4D5 isoform. These were confirmed by mutagenesis analyzed through both pull-down and two-hybrid studies (25). Here we have generated a library of overlapping peptides (25-mers), each shifted by 5 amino acids, which spans the entire sequence of βarrestin1. These were spot-synthesized on cellulose membranes to generate an immobilized peptide library that was then probed with a purified, recombinant GST fusion protein of active MEK1. Binding of MEK1 to individual peptide spots was assessed immunologically, with positive interactions identified as dark spots (Fig. 1a). A cluster of peptides (spots 3–6) was observed for GST-MEK1 binding but not GST alone, and that yielding the strongest signal (spot 6; Fig. 1a) was evaluated further.

In order to gain insight into the amino acids involved in allowing MEK1 to bind to this region of βarrestin1, we generated a family of peptides derived from the “spot 6” 25-mer parent peptide whose sequence reflected amino acids Asp26–Glu50 of βarrestin1. The 25-mer peptide progeny of this parent peptide each had a single substitution, to alanine, of successive amino acids in the sequence to form a scanning peptide array (Fig. 1b). Using this analysis, we observed that MEK1 binding to the 25-mer Asp26–Glu50 βarrestin peptide was ablated upon alanine substitution of Asp26 and Asp29 (Fig. 1b). Binding of MEK1 was also ablated when the various amino acids in the region Asp26 to His30 were all replaced with alanine residues but not when the run of amino acids from Tyr26 to Glu50 were similarly substituted with alanine residues.

To reinforce our findings from peptide array analysis, we attempted to show that MEK1 and βarrestin1 could directly interact in vitro. After mixing purified fusion proteins of His-MEK1 with GST-βarrestin1 or GST alone, we pulled down the His tag on MEK1 and evaluated co-immunoprecipitating species by Western blotting (Fig. 2a). GST-βarrestin1, but not GST, co-purified with His-MEK1, confirming the notion that the MEK1-βarrestin1 association is direct and is not dependent on other putative members of the signaling complex. In order to determine if this DXXD motif does indeed play an important role in defining the interaction between MEK1 and βarrestin, we mutated Asp26 and Asp29 to alanine in a FLAG epitope-tagged βarrestin1 construct and evaluated whether this altered the ability of MEK1 to co-immunoprecipitate with βarrestin in cell lysates where they are co-expressed (Fig. 2b). Doing this, we see that although anti-FLAG immunoprecipitates of FLAG-tagged βarrestin1 pulled down endogenous MEK1, only 8.3 ± 3.9% (mean; n = 3) of MEK remains associated with the FLAG-tagged D26A/D29A-βarrestin1 mutant construct (Fig. 2b). There is specificity in this interaction, since both the wild-type and D26A/D29A mutant forms of FLAG-tagged βarrestin1 acted to pull-down equal amounts of PDE4D (Fig. 2), whose binding site on βarrestin does not involve either Asp26 or Asp29.
were substituted with alanines. Endogenous motif crucial for MEK1 interaction (see above). This peptide, or all three basic residues (Arg and Asp) amino acids 6–29 of (Fig. 3) (Thr and Asp–6 underlined and Asp and Arg play a key role in underpinning each residue, indicated as boldface and underlined, within spot 10 (Gln–Glu–50 MEK1) were substituted with alanines and monitored for interaction with GST–βArrestin1 and was selected for alanine-scanning analysis. b, multiple residues, indicated as boldface and underlined, within spot 10 (Gln–Glu–50 MEK1) were substituted with alanines and monitored for interaction with GST–βArrestin1 as in a. c, HEK293 cells were dually transfected with FLAG–βArrestin1 and either HA-MEK1 or a mutant of HA-MEK1 in which Arg47, Lys48, Arg49 had been mutated to AAA. βArrestin1 was immunopurified using the FLAG epitope, and preparations were immunoblotted for the presence of HA-tagged MEK1. Ab, antibody.

βArrestin has also been shown to interact with ERK (10). Since MEK1 can interact with ERK, it has been postulated that Arrestin1 also led to loss of association with ERK of MEK1 in which Arg47, Lys48, Arg49 had been mutated to AAA. βArrestin1 was immunopurified using the FLAG epitope, and preparations were immunoblotted for the presence of HA-tagged MEK1. Ab, antibody.

In order to explore whether this cluster of positively charged amino acids in MEK1 is involved in the binding of βArrestin1, we generated a triple mutation of HA epitope-tagged MEK1, namely R47A/K48A/R49A-MEK1. In cells transfected with both FLAG-tagged βArrestin1 and HA-tagged MEK1, the kinase clearly co-immunoprecipitated with βArrestin1, as seen using anti-FLAG antiserum (Fig. 3c). In marked contrast to this, the triple mutant form, R47A/K48A/R49A-MEK1, failed to co-immunoprecipitate with βArrestin1 (Fig. 3c).

Use of a 25-Mer βArrestin Peptide to Disrupt MEK1–βArrestin Complexes in Cells—The “native” 25-mer peptide, TRVKKASPNKLTLYGKRDFVD29 (Thr6–Asp26–Phe27–Val28–Asp29) derived from βArrestin1 includes the Asp26–XX–Asp29 motif crucial for MEK1 interaction (see above). This peptide was modified by N-terminal stearoylation, which has been shown to allow entry of a variety of peptides of this size into cells so as to disrupt complexes (26, 33, 34). We also constructed a stearylated “mutant” peptide that had Asp26 and Asp29 each substituted with alanine, TRVKKASPNKLTLYGKR-AFVA29 (Thr6–Ala26–Phe27–Val28–Ala29). Two issues influenced our selection of this peptide. First, the fact that it contained only one DXXD motif, namely the critical one that when mutated causes loss of MEK binding, allowed us to undertake the “cleanest” experiment, where we focus on the key region, and also the “cleanest” control peptide, where this one motif.
**MEK1 Binds Directly to βArrestin1**

(a) βarr1 βarr1 WT D26-D-AA

![Image of Western blot results for FLAG-βarrestin1 and FLAG-βarrestin1-D26-D-AA](image)

(b) MEK displacement peptide

![Image of Western blot results for FLAG-βarrestin1 and FLAG-βarrestin1 with MEK displacement peptide](image)

(c) MEK Displacement

![Image of Western blot results for EGF treatment with MEK displacement peptide](image)
clear evidence of phosphorylation at such a site through analysis with a specific phosphoantiserum (Fig. 5). This event was mediated by ERK, since it was attenuated using a 4-h pretreatment of the MEK-selective inhibitors UO126 and PD98059 and a combination of both (Fig. 5a). However, no such phosphorylation was evident for FLAG-tagged D26A/D29A-βarrestin1, which does not bind MEK1 (Fig. 5a).

We then set out to explore whether the cell-permeable, stearoylated 25-mer βarrestin peptide (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹) that disrupts βarrestin-MEK1 complexes affected the ERK phosphorylation status of βarrestin1 in cells (Fig. 5b). Indeed, treatment of HEK293 cells with this peptide, but not with the control D26A/D29A-substituted one, led to a marked reduction in the Ser⁴¹² phosphorylation status of βarrestin1 (Fig. 5b). To counteract the notion that mutation of charged residues Asp²⁶/Asp²⁹ on the control peptide may differentially affect its entry into cells, fluorescein-labeled versions of both control and mutant peptides were compared for cell distribution (supplemental Fig. 1). From this we see (supplemental Fig. 1) that both peptides seemingly had the ability to accumulate in the membrane and cytosol of HEK cells to a similar degree. To further test the efficiency of the MEK disruptor peptide, we treated HEK cells with EGF to markedly increase the pool of phosphorylated, activated ERK available within the cells. Interestingly, the amount of Ser⁴¹² phosphorylation of βarrestin1 remained unchanged compared with control during peak EGF stimulation (5 min of EGF, vehicle control, second lane), indicating that βarrestin1 was maximally phosphorylated by ERK under basal conditions. Additionally, the efficiency of the disruptor peptide to facilitate the dephosphorylation of βarrestin1 on Ser⁴¹² was unaffected by EGF treatment (Fig. 5c, third panel), suggesting that disruption of the MEK-βarrestin1 complex induced by the displacement peptide is dominant even under conditions of elevated global ERK activity.

**The Importance of MEK1 Binding to βArrestin in Regulating the Association of Clathrin and Src to βArrestin**—It is well established that the phosphorylation of βarrestin1 at Ser⁴¹²
occurs when β-arrestin translocates to the agonist-occupied β₂-adrenergic receptor (11). This is believed to act as a trigger for clathrin-mediated receptor endocytosis, since dephosphorylation at Ser⁴¹² increases the affinity of β-arrestin for binding to clathrin and also to c-Src (9, 12). Here we show that upon isoprenaline challenge of HEK293 cells transiently overexpressing the β₂-adrenergic receptor and FLAG-tagged β-arrestin1, there is an increase in the association of both clathrin and c-Src with β-arrestin1 (Fig. 6). When these cells were treated with the MEK displacer peptide (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹), there was a marked increase in isoprenaline-induced association of β-arrestin1 with both clathrin and c-Src (Fig. 6). These findings are consistent with the notion that the peptide promotes dephosphorylation of β-arrestin1 at Ser⁴¹². Indeed, treatment with this MEK-displacing peptide seemed to promote association of c-Src and clathrin with β-arrestin1 in the basal, unstimulated cells. In contrast, the D26A/D29A-substituted “mutant” peptide failed to influence the binding of either clathrin or c-Src to β-arrestin1 under either basal or stimulated conditions (Fig. 6). Control vesicular stomatitis virus immunoprecipitations showed that neither FLAG-tagged β-arrestin1, c-Src, clathrin, nor ERK were pulled down in a non-specific manner (supplemental Fig. 2) by the agarose beads.

Displacement of the MEK1-β-Arrestin1 Complex Promotes β₂-AR Endocytosis—Plasma membrane-recruited β-arrestin, when dephosphorylated at Ser⁴¹², undergoes receptor-mediated endocytosis through its increased association with clathrin and c-Src. We followed isoprenaline-triggered β₂-AR endocytosis in HEK-B2 cells, which constitutively express β₂-ARs tagged with both FLAG and GFP (35). We did this by monitoring the internalized population of receptors in a biochemical assay and by confocal microscopy utilizing the GFP tag on the stably expressed receptor. First, we show,
MEK1 Binds Directly to βArrestin1

using an assay where the receptors are biotinylated, that treatment of the cells with the 25-mer β-arrestin peptide (Thr6–Asp26–Phe27–Val28–Asp29) that disrupts MEK1 binding acts to attenuate ERK-mediated Ser412 phosphorylation on β-arrestin1 and markedly facilitates the rate of isoprenaline-mediated internalization of the β2-AR (Fig. 7a). In contrast to this, treatment with the mutant D26A/D29A-substituted peptide did not.

When we visualized the isoprenaline elicited internalization of the β2-AR in these cells using confocal microscopy, an obvious (Fig. 7b) and significant (Fig. 7c) potentiation of receptor internalization was observed in the MEK1 binding disruptor peptide-treated but not the D26A/D29A-substituted mutant peptide-treated cells. Together, these findings support the notion that the displacement of the MEK1-β-arrestin1 complex attenuates the phosphorylation of Ser412 by ERK MAPKs and promotes β-arrestin1 association with clathrin and c-Src to enhance β2-AR internalization.

**DISCUSSION**

The phosphorylation of β-arrestin1 at Ser412 by ERK MAPK has been shown to regulate its endocytic properties but is not connected to the ability of β-arrestin to desensitize the β2-AR (9). Upon receptor activation, β-arrestin translocates to the plasma membrane, binds the phosphorylated receptor, and is dephosphorylated, a process that allows clathrin/c-Src binding followed by c-Src-mediated phosphorylation of dynamin that is essential for G-protein-coupled receptor endocytosis to ensue (36, 37). Interestingly, the control of the β-arrestin locality via phosphorylation/dephosphorylation is also seen in β-arrestin2, where phosphorylation occurs at different sites (Ser361 and Thr383) and by a different kinase (casein kinase II) (38, 39). Phosphorylation of β-arrestin1 at Ser412 can also be triggered by insulin, and this can block isoprenaline-induced dephosphorylation and subsequent β2-AR receptor internalization and downstream ERK signaling (40). In all examples, the phosphorylation of β-arrestin1 seems to be dependent on signaling via MEK, the ampidon for ERK, since either MEK inhibitors or arrestin (Thr6–Asp26–Thr27–Phe28–Ala29). The β2-ARs expressed in HEK293 cells were biotinylated before cells were treated with isoprenaline (10 μM) for 5 min. Receptors at the surface were stripped before cells were lysed and lysates were immunoblotted for internalized receptor using an antibody specific for the β2-AR.*, statistical significance (p < 0.05 using Student’s t test), b, HEK293 cells were similarly treated with peptide and control peptide as for a before being stimulated with isoprenaline (10 μM) for 5 min. Cells were then fixed and subjected to analysis by confocal microscopy visualizing the GFP tag on the stably transfected β2 ARs in these cells. c, quantification of internalized GFP-β2-AR done as described under “Experimental Procedures.” n = number of cells analyzed/treatment. ***p < 0.001.

**FIGURE 7.** Disruption of the MEK1-β-arrestin1 complex using a disruptor peptide promotes β2-AR endocytosis following isoprenaline treatment. a, HEK293 cells were pretreated for 2 h with vehicle (DMSO), a cell-permeable peptide (10 μM) that encompassed amino acids 6–29 of β-arrestin (Thr6–Asp26–Phe27–Val28–Asp29) or a mutant peptide (10 μM) where residues Asp26 and Asp29 were substituted with alanines (Thr6–Ala26–Phe27–Val28–Ala29). The β2-ARs expressed in HEK293 cells were biotinylated before cells were treated with isoprenaline (10 μM) for 5 min. Receptors at the surface were stripped before cells were lysed and lysates were immunoblotted for internalized receptor using an antibody specific for the β2-AR.*, statistical significance (p < 0.05 using Student’s t test), b, HEK293 cells were similarly treated with peptide and control peptide as for a before being stimulated with isoprenaline (10 μM) for 5 min. Cells were then fixed and subjected to analysis by confocal microscopy visualizing the GFP tag on the stably transfected β2-ARs in these cells. c, quantification of internalized GFP-β2-AR done as described under “Experimental Procedures.” n = number of cells analyzed/treatment. ***p < 0.001.
MEK1 binds directly to β-arrestin1

Indeed, it is well appreciated that β-arrestin1 can undergo structural changes upon post-translational modification and binding to receptors (44, 45). Thus, we would suggest that MEK probably binds to either a modified or complexed subpopulation of β-arrestin1 that allows exposure of Asp26 and Asp29 in its N-terminal region. Another possibility is that β-arrestin1 may exist in equilibrium between its basal state and a low abundance “open conformation” resembling that to which MEK, and perhaps the phospho-GPCR, can bind. If so, MEK might bind to the open conformation, stabilizing this and shifting the equilibrium toward to form a β-arrestin1 pool complexed by MEK. It is noteworthy that the Asp26- and Asp29-containing sequence will be prominently surface-exposed within the isolated β-arrestin N-domain. Therefore, the ability of this isolated domain to bind MEK1 (24) would be consistent with an interaction mediated, at least in part, by these two aspartate residues. Furthermore, if, as Song et al. (24) suggest, there is an interaction of MEK1, either directly or indirectly, with the C-domain, then this may act as the trigger to expose Asp26 and Asp29 at the N-domain of β-arrestin for MEK1 binding.

We have also used peptide array analysis to determine the sites on MEK that associate with β-arrestin1. The accessibility of the identified β-arrestin-binding arginines, Arg47 and Arg49, of MEK1 is not known, because they are absent in available crystal structures (46). However, their localization in the N terminus

upstream of the kinase domain would be consistent with the notion that they may be surface-exposed and thereby available for interaction.

The identification of the MEK-binding motif on β-arrestin1 has allowed the generation of double-substituted alanine mutants of β-arrestin1 that cannot bind with MEK1. We have also used a novel small molecule technique in order to develop a cell-permeable peptide inhibitor of MEK1 binding to β-arrestin1 and used this to show that MEK1 binding to β-arrestin1 regulates the phosphorylation of β-arrestin1 at the established ERK phosphorylation site at Ser412 even in times of heightened global ERK activity. Lack of direct MEK1 binding to the β-arrestin1-ERK complex thus decreases the phosphorylation levels of β-arrestin1, which allows β-arrestin1 to bind more readily to clathrin and c-Src, integral parts of the endocytic machinery. This augmented association of β-arrestin1 with clathrin/c-Src induces an increase in β2-AR internalization, a process known to be initiated by dephosphorylated β-arrestin at Ser412 (11). Indeed, the fact that the MEK disruptor peptide induces β-arrestin1 dephosphorylation, heightens the interaction of β-arrestin1 with c-Src/clathrin, and promotes receptor internalization strongly suggests that the dephosphorylation of β-arrestin1 at Ser412 is a rate-limiting step for the receptor internalization process.

We have presented data that represent the first identification of the MEK1-binding site on β-arrestin1. In mapping the binding sites on both MEK1 for β-arrestin1 and on β-arrestin1 for MEK1, we have generated mutants that disrupt the interaction of these two components while not affecting ERK association. Additionally, we have been able to design and apply a small peptide that disrupts the MEK1/β-arrestin1 interaction in cells to facilitate study of the functional consequences of this interaction. This disruption of MEK1 association has clear implications for ERK action on β-arrestin1, since it ablates ERK-mediated phosphorylation of β-arrestin1 and alters ERK-mediated regulation of the internalization and recycling of the β2-AR. Such a peptide has thus potential for manipulation of the internalization and recycling of the β2-AR.

REFERENCES
1. Defea, K. (2008) Br. J. Pharmacol. 153, Suppl. 1, 298–309
2. DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007) Annu. Rev. Physiol. 69, 483–510
3. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) Can. J. Physiol. Pharmacol. 74, 1095–1110
4. Premont, R. T., and Gainetdinov, R. R. (2007) Annu. Rev. Physiol. 69, 511–534
5. Bairle, G. S., Sood, A., McPhee, I., Gall, I., Perry, S. J., Lefkowitz, R. J., and Houslay, M. D. (2003) Proc. Natl. Acad. Sci. U. S. A 100, 940–945
6. Gurevich, V. V., and Gurevich, E. V. (2006) Pharmacol. Ther. 110, 465–502
7. Bolger, G. B., Bairle, G. S., Li, X., Lynch, M. J., Herzyk, P., Mohamed, A., Mitchell, L. H., McCallil, A., Hundsrucker, C., Klussmann, E., Adams, D. R., and Houslay, M. D. (2006) Biochem. J. 398, 23–36
8. Lynch, M. J., Bairle, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G., and Houslay, M. D. (2005) J. Biol. Chem. 280, 33178–33189
9. Lin, F. T., Miller, W. E., Luttrell, L. M., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 15971–15974
10. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A 98, 2449–2454
11. Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 31051–31057
12. Miller, W. E., Maudsley, S., Ahn, S., Khan, K. D., Luttrell, L. M., and Lefkowitz, R. J. (2000) J. Biol. Chem. 275, 11312–11319
13. Tanoue, T., and Nishida, E. (2002) Pharmacol. Ther. 93, 193–202
14. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) Nat. Cell Biol. 2, 110–116
15. Pelaia, G., Cuda, G., Vatrella, A., Gallelli, L., Caraglia, M., Marra, M., Abbruzzese, A., Caputi, M., Maselli, R., Costanzo, F. S., and Marsico, S. A. (2005) J. Cell. Physiol. 202, 642–653
16. English, J. M., and Cobb, M. H. (2002) Trends Pharmacol. Sci. 23, 40–45
17. Sweeney, S. E., and Firestein, G. S. (2006) Annu. Rheum Dis 65, Suppl. 3, 83–88
18. Lefkowitz, R. J., Inglese, J., Koch, W. J., Ohra, S., and Caron, M. G. (1992) Cold Spring Harb. Symp. Quant. Biol. 57, 127–133
19. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) J. Cell Biol. 148, 1267–1281
20. McDonald, P. H., and Lefkowitz, R. J. (2001) Cell. Signal. 13, 683–689
21. Perry, S. J., and Lefkowitz, R. J. (2002) Trends Cell Biol. 12, 130–138
22. Lefkowitz, R. J., and Whalen, E. J. (2004) Curr. Opin. Cell Biol. 16, 162–168
23. Lefkowitz, R. J., and Shenoy, S. K. (2005) Science 308, 512–517
24. Song, X., Coffa, S., Fu, H., and Gurevich, V. V. (2008) J. Biol. Chem.
25. Bolger, G. B., McCahill, A., Huston, E., Cheung, Y. F., McSorley, T., Bairle, G. S., and Houslay, M. D. (2003) J. Biol. Chem. 278, 49230–49238
26. Futaki, S., Ohashi, W., Suzuki, T., Niwa, M., Tanaka, S., Ueda, K., Harashima, H., and Sugii, R. (2001) Bioconjugate Chem. 12, 1005–1011
27. Bairle, G. S., Adams, D. R., Bari, N., Houslay, T. M., Vadrevu, S., Meng, D., Li, X., Dunlop, A., Milligan, G., Bolger, G. B., Klussmann, E., and Houslay, M. D. (2007) Biochem. J. 404, 71–80
28. Catling, A. D., Schaeffer, H. I., Reuter, C. W., Reddy, G. R., and Weber, M. J. (1995) Mol. Cell. Biol. 15, 5214–5225
29. Mackenzie, S. J., and Houslay, M. D. (2000) Biochem. J. 347, 571–578
30. Shepherd, M. C., Baillie, G. S., Stirling, D. L., and Houslay, M. D. (2004) Br. J. Pharmacol. 142, 339–351
31. Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., and Luttrell, L. M. (2002) J. Biol. Chem. 277, 9429–9436
32. Xu, T. R., Baillie, G. S., Bhar, N., Houslay, T. M., Pitt, A. M., Adams, D. R., Kolch, W., Houslay, M. D., and Milligan, G. (2008) Biochem. J. 413, 51–60
33. Smith, K. J., Baillie, G. S., Hyde, E. I., Li, X., Houslay, T. M., McCahill, A., Dunlop, A. J., Bolger, G. B., Klussmann, E., Adams, D. R., and Houslay, M. D. (2007) Cell. Signal. 19, 2612–2624
34. Murdoch, H., Mackie, S., Collins, D. M., Hill, E. V., Bolger, G. B., Klussmann, E., Porteous, D. J., Millar, J. K., and Houslay, M. D. (2007) J. Neurosci. 27, 9513–9524
35. Li, X., Huston, E., Lynch, M. J., Houslay, M. D., and Baillie, G. S. (2006) Biochem. J. 394, 427–435
36. Ahn, S., Maudsley, S., Luttrell, L. M., and Daaka, Y. (1999) J. Biol. Chem. 274, 1185–1188
37. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Science 283, 655–661
38. Kim, Y. M., Barak, L. S., Caron, M. G., and Benovic, J. L. (2002) J. Biol. Chem. 277, 16837–16846
39. Lin, F. T., Chen, W., Shenoy, S., Cong, M., Exum, S. T., and Lefkowitz, R. J. (2002) Biochemistry 41, 10692–10699
40. Hupfeld, C. J., Resnik, J. L., Ugi, S., and Olefsky, J. M. (2005) J. Biol. Chem. 280, 1016–1023
41. Han, M., Gurevich, V. V., Vishnivetskiy, S. A., Sigler, P. B., and Schubert, C. (2001) Structure 9, 869–880
42. Milano, S. K., Kim, Y. M., Stefano, F. P., Benovic, J. L., and Brenner, C. (2006) J. Biol. Chem. 281, 9812–9823
43. Xiao, K., McClatchy, D. B., Shukla, M. K., Zhao, Y., Chen, M., Shenoy, S. K., Yates, J. R., III, and Lefkowitz, R. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 12011–12016
44. Gurevich, V. V., and Gurevich, E. V. (2003) Structure 11, 1037–1042
45. Vishnivetskiy, S. A., Hosey, M. M., Benovic, J. L., and Gurevich, V. V. (2004) J. Biol. Chem. 279, 1262–1268
46. Ohren, J. F., Chen, H., Pavlovsky, A., Whitehead, C., Zhang, E., Kuffa, P., Yan, C., McConnell, P., Spessard, C., Banotai, C., Mueller, W. T., Delaney, A., Omer, C., Sebolt-Leopold, J., Dudley, D. T., Leung, I. K., Flamme, C., Warmus, J., Kaufman, M., Barrett, S., Tecle, H., and Haseman, C. A. (2004) Nat. Struct. Mol. Biol. 11, 1192–1197