MEK1 Binds Directly to β Arrestin1, Influencing Both Its Phosphorylation by ERK and the Timing of Its Isoprenaline-stimulated Internalization

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β Arrestin is a multifunctional signal scaffold protein. Using SPOT immobilized peptide arrays, coupled with scanning alanine substitution and mutagenesis, we show that the MAPK kinase, MEK1, interacts directly with β Arrestin1. Asp26 and Asp29 in the N-terminal domain of β Arrestin1 are critical for its binding to MEK1, whereas Arg27 and Arg29 in the N-terminal domain of MEK1 are critical for its binding to β Arrestin1. Wild-type FLAG-tagged β Arrestin1 co-immunopurifies with MEK1 in HEK2B cells, whereas the D26A/D29A mutant does not. ERK-dependent phosphorylation at Ser412 was compromised in the D26A/D29A-β Arrestin1 mutant. A cell-permeable, 25-mer N-stearylated β Arrestin1 peptide that encompassed the N-domain MEK1 binding site blocked β Arrestin1/MEK1 association in HEK cells and recapitulated the altered phenotype seen with the D26A/D29A-β Arrestin1 in compromising the ERK-dependent phosphorylation of β Arrestin1. In addition, the MEK disruptor peptide promoted the ability of β Arrestin1 to co-immunoprecipitate with endogenous c-Src and clathrin, facilitating the isoprenaline-stimulated internalization of the β2-adrenergic receptor.

The β Arrestins 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 β Arrestins (3, 4). By associating with agonist-occupied receptors, β Arrestins 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 β Arrestin with clathrin cages provides a key part of the paradigm for the deactivation of select ligand-boundGPCRs (6). β Arrestins can also deliver sequestered cAMP phosphodiesterase-4 isofoms, 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 β Arrestin1 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 β Arrestin to GPCRs, such as the β2-AR, leads to the dephosphorylation of β Arrestin1 at this site. This event acts as a molecular switch, allowing for the internalization of the β2-AR-sequestered β Arrestin complex. Thus, ERK-phosphorylated β Arrestin1 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 β Arrestin1 with c-Src and the phosphorylation of dynamin, a key feature in receptor internalization (12). Thus, the ERK2-dependent Ser412 phosphorylation and dephosphorylation of β Arrestin1 provides a pivotal molecular switch that determines the association of β Arrestin1 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.
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and asthma (15–17). It has previously been suggested that MEK may form a complex with ERK and with βarrestin (10, 18) that creates a "signalosome" capable of disseminating MAPK signals to defined intracellular compartments. In other studies, it has been suggested that constitutive βarrestin-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 βarrestin1 (24) within sites on both N and C domains. Critically, however, the exact nature of MEK interaction with βarrestin1 is unclear. Given that βarrestin1 is ERK-phosphorylated in the cytosol (9) and the critical importance of this event to βarrestin-mediated GPCR internalization, the nature of MEK1 interaction with βarrestin warrants investigation. Here then, we evaluate MEK1 interaction with the signal scaffolding protein, βarrestin1, demonstrating that MEK1 binds directly to βarrestin1, 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 βarrestin1 phospho-Ser112 (2416) (Cell Signaling Technology, Beverly, MA); β2-AR (sc-569), glutathione S-transferase (GST) (sc-53909), clathrin HC (sc-12734), and hemagglutinin probe (sc-7372) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); βarrestin1 phospho-Ser112 (44-200; lot 0102), anti-Src pan (44-655G), and anti-His-horseradish peroxidase (P/N 56-0707) (Invitrogen); and FLAG-horseradish peroxidase (A8592) (Sigma). Monoclonal antisera 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) were purchased from Millipore. HEKB2 cells were a kind gift from Prof. Graeme Milligan (University of Glasgow). Cell-permeable peptides were a gift from Prof. Enno Klusmann (Leibniz-Institut für Molekulare Pharmakologie, Berlin).

Cell Culture and Drug Additions—HEK293 cells and HEK293 cell lines stably overexpressing FLAG-tagged β2-AR-GFP (HEKB2 cells) were cultured as described previously (25). Transfection of these cells was done using Polyfect (Qiagen), following the manufacturer’s instructions. Pretreatments for control experiments were done 10 min (UO126, 10 μM) or 2 h (cell-permeable peptides, 10 μM) prior to the addition of isoproterenol (10 μM).

Preparation of Peptide Small Molecule Disruptors—Briefly, all stearil-peptides were prepared by treatment of the peptide resins with stearic acid and diisopropylcarbodiimide in the presence of N-hydroxysuccinimide, followed by deprotection using trifluoroacetic acid/ethanedithiol (26).

Fluorescent peptides were prepared by adding 2 mg of 5,6-carboxyfluorescein N-hydroxysuccinimide ester (in 0.9 ml of DMSO, 100 μl of 0.1 x NaHCO3, pH 8.5) to 10 mg of peptide to be labeled. After reaction at room temperature in darkness for 20 h, the reaction mix was directly applied to preparative high pressure liquid chromatography. Fractions containing labeled peptide were collected and lyophilized.

Peptide Arrays and Alanine Scans—βarrestin1 and MEK1 peptide libraries were produced by automatic SPOT synthesis as described previously (27). They were synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc (N-(9-fluorenly)methoxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany). Alanine-scanning peptide libraries were constructed by taking the residues in positive spots and sequentially changing each residue to alanine (or, if an alanine was the natural amino acid at that position, to aspartate). The interaction of spotted peptides with purified, recombinant GST and GST-βarrestin1 and GST-MEK1 fusion proteins was determined by overlaying the cellulose membranes with 10 μg/ml recombinant protein. Bound recombinant proteins were then detected following wash steps with rabbit anti-GST, and detection was performed with a secondary anti-rabbit horseradish peroxidase-coupled antibody.

Expression of GST Fusions in Escherichia coli—Cultures of E. coli JM109 containing pGEX-β-arrestin1 or pGEX were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Roche Applied Science) for 4 h at 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 βarrestin1, which was generated by an insertion of the full open reading frame of βarrestin1 into the Not1 site of pcDNA3 (Invitrogen), was a kind gift from Dr. Graeme B. Bolger (University of Alabama at Birmingham). Primers 5′-GGAGCAGGCAGGCGGCTGACCCAGGATCAGCTCA-3′ and 5′-GGTCCAGAAGGGCGAGGATCGCTGAGCGCGATGAC-3′ were used to generated the FLAG-tagged D26A/D29A-βarrestin1 mutant with the Quik-Change site-directed mutagenesis kit (Stratagene). The pCHA-MEK1 construct was a gift from Prof. Walter Kolch (Beatson Institute for Cancer Research, Glasgow, UK). Details of the construct were described previously (28). Primers 5′-GGGAGCAGGCAGGCGGCTGACCCAGGATCAGCTCAAG-3′ and 5′-CGTCGAGAAGGGCGAGGATCGCTGAGCGCGATGAC-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)
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with bovine serum albumin as a standard using Bradford reagent as previously described (25). Protein was routinely measured by the method of Bradford using bovine serum albumin as a standard.

Immunoprecipitation of Target Molecules—This was done as described previously by us (29, 30). Briefly, detergent-soluble proteins were isolated from cells by disruption in lysis buffer (1% (v/v) Triton X-100, 50 mM HEPES buffer, pH 7.2, 10 mM EDTA, 100 mM NaH₂PO₄, 2H₂O) containing complete protease inhibitor mixture (Roche Applied Science) to 8% volume. Detergent-insoluble proteins were removed by centrifugation at 10,000 \( \times g \) for 10 min, and the soluble fraction was retained. Equal volumes of cell lysate containing 500 \( \mu \)g of protein were cleared by incubation with 30 \( \mu \)l of preimmune serum and/or 30 \( \mu \)l of protein A slurry. The beads were then removed by centrifugation at 10,000 \( \times g \) for 10 min at 4°C, and cleared lysate was incubated at 4°C for 2 h with constant agitation with a volume of antiserum. Immunoglobulins were then isolated by incubation with protein A-coated Sepharose beads for 1 h before retrieval by refrigerated centrifugation at 10,000 \( \times g \) for 5 min. Target molecule-immunoglobulin conjugates attached to the beads were then washed in phosphate-buffered saline (PBS) three times.

Internalization of \( \beta \)-AR Assay—Briefly, cells were grown to 80% confluence in Petri dishes and treated with 0.3 mg/ml disulfide-liable 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 mM glutathione, 0.3 mM NaCl, 0.075 mM 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-\( \beta \)-AR antisera (sc-569; Santa Cruz Biotechnology).

Analysis by Microscopy—Slides were examined using a fluorescent imaging microscope at a magnification of \( \times 43 \) and imaged for phase contrast, GFP fluorescence, and \( 4',6'-dia- 

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-\( \beta \)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 \( \mu \)l of anti-polyhistidine-agarose beads (A5713; Sigma) were added in for an overnight incubation. Beads were collected by 10,000 \( \times g \) 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 \( \mu \)g of cellular protein/well. After treatment, HEK\( \beta \)2 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 with bovine serum albumin as a standard using Bradford reagent as previously described (25). Protein was routinely measured by the method of Bradford using bovine serum albumin as a standard.

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Analysis by Microscopy—Slides were examined using a fluorescent imaging microscope at a magnification of \( \times 43 \) and imaged for phase contrast, GFP fluorescence, and 4',6-dia- 

$\text{FIGURE 1. MEK1 binds directly to N domain of } \beta \text{arrestin1 peptide array.} \text{ a, immobilized peptide spots of overlapping 25-mer peptides, each shifted by 5 amino acids, covering the entire sequence of } \beta \text{arrestin1 were probed for interaction with either GST-MEK1 or GST alone by immunoblotting. Positively interacting peptides are represented by dark spots. Spot 6 covering the sequence Asp26–Glu50 produced the greatest interaction with GST-MEK1 and was selected for alanine-scanning analysis. b, each amino acid from spot 6 was sequentially and individually substituted with an alanine residue and overlaid with GST-MEK as in a. Control represents an identical spot to } \beta \text{arrestin1 peptide array.}$

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

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midino-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-adrenergic receptor. 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 isomorph. 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 Tyr46 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 blot analysis for the presence of both GST and His tags. b, HEK293 cells were transfected with FLAG-βarrestin1 or a mutant form of βarrestin1 where Asp26 and Asp29 had been dually mutated to alanine. Both forms of βarrestin1 were immunopurified using the FLAG epitope, and preparations were immunoblotted for MEK, ERK, and PDE4D. The lower panel shows transfection efficiency of the βarrestin1 constructs. Ab, antibody.
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FIGURE 3. βArrestin1 binds to the N-terminal of MEK1 at residues Arg47–Lys70. a, immobilized peptide spots of overlapping 25-mer peptides, each shifted by 5 amino acids, covering the entire sequence of MEK1 were probed for interaction with either GST-βArrestin1 or GST alone by immunoblotting. Positively interacting peptides are represented by dark spots. Spot 10, covering the sequence Gln46–Glu50, produced the greatest interaction with βArrestin1 and was selected for alanine-scanning analysis. b, multiple residues, indicated as boldface and underlined, within spot 10 (Gln46–Glu50 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–Lys70 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.

(27). These analyses confirm the peptide array studies in indicating that both Asp26 and Asp29 play a key role in underpinning the interaction of MEK1 with βArrestin1.

βArrestin has also been shown to interact with ERK (10). Since MEK1 can interact with ERK, it has been postulated that ERK may act as an adaptor that, in binding to βArrestin, sequesters MEK1 there. We thus set out to determine if loss of MEK1 association with FLAG-tagged D26A/D29A-βArrestin1 also led to loss of association of ERK with βArrestin1 (Fig. 2). Doing this, we see quite clearly that this is not the case (Fig. 2). Indeed, if anything, we noted an increase in association of ERK with the “cleanest” experiment, where we focus on the key region, Arg47 and Lys48 or all three basic residues (Arg47, Lys48, and Arg49) for alanine ablated the interaction of this mutant form of βArrestin1 with MEK1. In contrast, substitution of an acidic cluster, Asp65, Asp66, and Asp67, had little effect (Fig. 3b).

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 antisera (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, 6TRVKKASPNGKLTVYGKRDVFV (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, 6TRVKKASPNGKLTVYGKR–AFVA (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
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(a) βarr1 βarr1 WT D26-D-AA

FLAG-βarrestin1

** **

(b) MEK displacement peptide

0 2hr 4hr

FLAG-βarrestin1 P-Ser 412

FLAG-βarrestin1

Control peptide

0 2hr 4hr

FLAG-βarrestin1 P-Ser 412

FLAG-βarrestin1

(c) MEK Displacement

| Vehicle | Cont. peptide |
|---------|---------------|
| EGF (min) | 0 | 5 | 30 | 0 | 5 | 30 | 0 | 5 | 30 |

PERK 1/2
ERK 1/2
Pser 412
FLAG

Phospho-Ser 412
FLAG
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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 U0126 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 ERK Phosphorylation Status of βArrestin1**—In cells that have not been stimulated by G-protein receptor agonists, βArrestin resides primarily in the cytoplasm, where it is constitutively phosphorylated at Ser⁴¹² through the action of ERK. Expression of our FLAG-tagged wild-type βArrestin1 showed (DXXD) could be changed, in this instance to AXAX. Also, the stearate group, which allows cell insertion, is added to the N-terminal end of the peptide, and we wished this to be as far as possible from the critical DXXD motif so as not to cause any possible steric interference.

In cells transfected to express FLAG-tagged βArrestin1, we see here that treatment with the native stearoylated 25-mer peptide (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹) ablated the co-immunoprecipitation of endogenous MEK1 with FLAG-tagged βArrestin1 (Fig. 4a). However, no such disruption of βArrestin-MEK1 complexes in cells was evident upon use of the control “mutant” 25-mer stearoylated peptide (Thr⁶–Ala²⁶–Phe²⁷–Val²⁸–Ala²⁹), where the MEK1 interaction site was disrupted by dual D26A/D29A substitution. The amount of MEK that could be immunoprecipitated with endogenously expressed βArrestin was at the edge of detection by our antibodies (only 4.7 ± 2.4% (mean; n = 3)); however, this association was attenuated (1.3 ± 1.9% (mean; n = 3)) using the native displacer peptide (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹) (Fig. 4b) but not the mutant peptide (4.2 ± 3.2% (mean; n = 3)).

The Importance of MEK1 Binding to βArrestin in Regulating the ERK Phosphorylation Status of βArrestin1—In cells that have not been stimulated by G-protein receptor agonists, βArrestin resides primarily in the cytoplasm, where it is constitutively phosphorylated at Ser⁴¹² through the action of ERK (9). Expression of our FLAG-tagged wild-type βArrestin1 showed

| Vehicle control | MEK displacer peptide | Control peptide |
|-----------------|-----------------------|-----------------|
| 0 min           | Lysate lp            | Lysate lp       |
| 5 min           | Lysate lp            | Lysate lp       |

**FIGURE 5.** Constitutive phosphorylation of βArrestin1 at serine 412 is attenuated following disruption of its association with MEK1. a, HEK293 cells were transfected with FLAG-βArrestin1 plus or minus the MEK inhibitors U0126 and PD98059 or a combination of both (1 μM for 4 h) or a mutant form of βArrestin1, where Asp26 and Asp29 had been dually mutated to alanine (D26A/D29A). Cell lysates were immunoblotted for expression of FLAG-βArrestin1 using the FLAG epitope or immunoblotted for phosphorylation at Ser⁴¹² on βArrestin1 using a site-directed phosphoantiserum. NT, no treatment. b, HEK293 cells were transfected with FLAG-βArrestin1 and treated with cell-permeable peptides (10 μM) that encompassed amino acids 6–29 (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹) of βArrestin, one of which had Asp²⁶ and Asp²⁹ mutated to alanine (Control peptide). Cell lysates were immunoblotted for expression of FLAG-βArrestin1 using the FLAG epitope or immunoblotted for phosphorylation at serine 412 on βArrestin1 using a site directed phosphoantibody. **, statistical significance (p < 0.01 using Student’s t test). c, HEK293 cells were transfected with FLAG-βArrestin1 before treatment (2 h) with cell-permeable peptides (10 μM) that encompassed amino acids 6–29 (Thr⁶–Asp²⁶–Phe²⁷–Val²⁸–Asp²⁹) of βArrestin, one of which had Asp²⁶ and Asp²⁹ mutated to alanine (Control peptide). Cell lysates were then treated with EGF (1 μM) for the indicated times. Cell lysates were immunoblotted for expression of FLAG-βArrestin1 and phosphorylation at serine 412 on βArrestin1, ERK, and phospho-ERK using appropriate antibodies. Wt, wild type.

**FIGURE 6.** Disruption of the MEK1-βArrestin1 complex using a disruptor peptide enhances association of βArrestin1 with c-Src and clathrin. HEK293 cells were transfected with β₂-AR and FLAG-βArrestin1 before being pretreated for 2 h with vehicle (DMSO), a cell-permeable stearoylated peptide (10 μM) that encompassed amino acids 5–29 of βArrestin1, or a mutant peptide (10 μM) where residues Asp26 and Asp29 were substituted with Ala. Cells were then stimulated with isoprenaline (10 μM) for 5 min before βArrestin1 was immunopurified using the FLAG epitope, and preparations were immunoblotted for FLAG, MEK, ERK, PDE4D, c-Src, and clathrin heavy chain.

**FIGURE 4.** A. Also, the (DXXD) motif so as not to cause any possible steric interference. D XX

Also, the (DXXD) motif so as not to cause any possible steric interference.
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occurs when βarrestin 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 βarrestin 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 βarrestin1, there is an increase in the association of both clathrin and c-Src with βarrestin1 (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 βarrestin1 with both clathrin and c-Src (Fig. 6). These findings are consistent with the notion that the peptide promotes dephosphorylation of βarrestin1 at Ser412. Indeed, treatment with this MEK-displacing peptide seemed to promote association of c-Src and clathrin with βarrestin1 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 βarrestin1 under either basal or stimulated conditions (Fig. 6). Control vesicular stomatitis virus immunoprecipitations showed that neither FLAG-tagged βarrestin1, c-Src, clathrin, nor ERK were pulled down in a non-specific manner (supplemental Fig. 2) by the agarose beads.

Displacement of the MEK1-βArrestin1 Complex Promotes β2-AR Endocytosis—Plasma membrane-recruited βarrestin, 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,
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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 of βarrestin1 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-βarrestin1 complex attenuates the phosphorylation of Ser412 by ERK MAPks and promotes βarrestin1 association with clathrin and c-Src to enhance β2-AR internalization.

**DISCUSSION**

The phosphorylation of βarrestin1 at Ser412 by ERK MAPK has been shown to regulate its endocytic properties but is not connected to the ability of βarrestin to desensitize the β2-AR (9). Upon receptor activation, βarrestin 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 βarrestin locality via phosphorylation/dephosphorylation is also seen in βarrestin2, where phosphorylation occurs at different sites (Ser361 and Thr383) and by a different kinase (casein kinase II) (38, 39). Phosphorylation of βarrestin1 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 βarrestin1 seems to be dependent on signaling via MEK, the amipor for ERK, since either MEK inhibitors or dominant negative inactive MEK attenuated modification at Ser412 (10, 31, 40). In this study, we demonstrate that βarrestin1 is largely phosphorylated by ERK at Ser412 and that this pool of ERK is activated by βarrestin1-associated MEK.

Here, we show that MEK1 can bind directly to βarrestin1 and that this association does not require any complexing to ERK in order to occur. Previous studies had indicated that MEK, ERK, and c-RAF1 can simultaneously associate with βarrestin, and, on this basis, it was tentatively suggested that c-RAF1 might act as a scaffold for MEK and ERK to facilitate their association with βarrestin (10, 31). Furthermore, during revision of this paper, utilizing transfected truncates of βarrestin1 and βarrestin2, Song et al. (24) suggested that MEK 1 may bind to both the N and C domains of βarrestins. In agreement, we demonstrate that MEK can directly bind to βarrestin1 at a docking site situated within the N-domain of βarrestin1. Interestingly, a recent report has identified a docking site for ERK on βarrestin that is clearly distinct from that identified here for MEK1, since it resides in the βarrestin1 C-domain (32). In light of all of these reports, it seems likely that βarrestin may directly scaffold ERK (MAPK), MEK (MAPK kinase), and c-RAF1 (MAPK kinase kinase) in order to orient each of them correctly for efficient downstream signaling. Our studies and those of Song et al. (24) may imply that there is a predominant direct interaction of MEK with the N-domain of βarrestin together with a subsidiary indirect interaction with the βarrestin C-domain achieved via an interaction of MEK with ERK. The binding sites for c-RAF on βarrestin, however, have yet to be elucidated.

The MEK1-βarrestin heterodimer is known to regulate the endocytic properties of βarrestin via ERK phosphorylation, which has downstream implications on the rate and amount of internalized β2-AR within the cell. Peptide array technology has allowed us to characterize specific βarrestin residues that play a role in the docking of MEK1, and scanning substitution arrays have identified a stretch of residues that contain a MEK1 binding motif (14), including amino acids Asp26 and Asp29 (Fig. 8). Interestingly, Asp26 and Asp29 have partial surface exposure in the basal arrestin conformation (Protein Data Bank codes 1G4M and 1G4R (41) and 1ZSH (42)), but they are essentially sequestered by interactions with adjacent residues. Asp26 forms salt bridges to Lys155 and Arg393 in the C-terminal sequence and also contacts the “phosphate sensor” residue, Arg169, whereas Asp29 forms a salt bridge to Lys170 and hydrogen-bonds to the side chain of Gln172 (Fig. 8). This makes it rather unlikely that MEK binds to these residues in βarrestin1 in the basal conformation identified by crystallography for βarrestin without any liganded partner proteins (Fig. 8). The involvement of Asp26 in binding of MEK is only possible if these residues are freed from their sequestered engagement by the arrestin C-terminal sequence. Displacement of the C-terminal tail sequence leaves Asp26 more exposed, but further conformational change must occur in order for Asp26 and Asp29 to be available to bind MEK. There is a paradigm for this in that arrestins can undergo a profound conformational change upon binding to their activated phosho-GPCR partners. This involves GPCR-phosphate interaction with the arrestin phosphate sensor and unlatching of the C-terminal tail sequence from the position it occupies in the basal conformation, folded across the N-terminal domain. It is known that βarrestin1 can bind to a myriad of partner proteins (21, 43), some of which are highly likely to cause structural alterations in this signaling scaffold protein.

**FIGURE 7.** Disruption of the MEK1-βarrestin1 complex using a disruptor peptide promotes β2-AR endocytosis following isoprenaline treatment. a, HEKB2 cells were pretreated for 2 h with vehicle (DMSO), a cell-permeable peptide (10 μM) that encompassed amino acids 6–29 of βarrestin (Thr6–Asp26–Phe27–Val28–Asp29) or a mutant peptide (10 μM) where residues Asp29 and Asp26 were substituted with alanines (Thr6–Ala26–Phe27–Val28–Ala29). The β2-ARs expressed in HEKB2 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, HEKB2 cells were similarly treated with peptide and control peptide as 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.
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Indeed, it is well appreciated that βarrestin1 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 βarrestin1 that allows exposure of Asp26 and Asp29 in its N-terminal region. Another possibility is that βarrestin1 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 through to form a βarrestin1 pool complexed by MEK. It is noteworthy that the Asp26- and Asp29-containing sequence will be prominently surface-exposed within the isolated βarrestin 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 βarrestin for MEK1 binding.

We have also used peptide array analysis to determine the sites on MEK that associate with βarrestin1. The accessibility of the identified βarrestin-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 βarrestin1 dephosphorylation, heightens the interaction of βarrestin1 with c-Src/clathrin, and promotes receptor internalization strongly suggests that the dephosphorylation of βarrestin1 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 βarrestin1. In mapping the binding sites on both MEK1 for βarrestin1 and on βarrestin1 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/βarrestin1 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 βarrestin1, since it ablates ERK-mediated phosphorylation of βarrestin1 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.

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On page 30551, Table 1, first column, the experimentally determined values of EC50 of ER activation for MC, Polysantol, Javanol, and androst enol have been presented with the wrong concentration (mm). The correct concentration is μM.

**TABLE 1**

Estrogenic activities of odorants

The compound concentrations (EC50) evoking half-maximal estrogenic activity in transcription activation, the dissociation inhibition constants (Ki) calculated from the IC50 values of the ES2 displacement curves (Fig. 1), and the corresponding Kd values calculated using the 6D-QSAR model are compared. For Polysantol and Javanol, only the Ki values for the energetically most favorable conformation are reported. The Ki values for the remaining stereoisomers are as follows: 140 ± 173 μM Polysantol (S,S), 216 ± 122 μM Polysantol (S,R), 2.86 ± 4.45 mM Polysantol (R,R), 2.41 ± 2.64 mM Javanol (R,S), 176.7 ± 567 μM Javanol (S,S), and 40.73 ± 23.33 μM Javanol (S,R). The Ki values for the remaining stereoisomeric forms are as follows: 299 μM Polysantol (S,S), 83 μM Polysantol (S,R), 1 mM Polysantol (R,R), 931 μM Javanol (R,S), 68 μM Javanol (S,S), and 15 μM Javanol (S,R).

| Compound       | EC50 of ER activation | Ki of ER competitive binding | Ki of ER predicted |
|----------------|------------------------|------------------------------|--------------------|
| Estradiol      | 0.16 ± 0.01 nM         | 1.6 ± 1 nM                   | 2 nM               |
| MC             | 86 ± 8 μM              | 76 ± 6 μM                    | 21 μM              |
| Polysantol (R,S)* | 98 ± 23 μM            | 56 ± 5 μM                    | 26 μM              |
| Javanol (R,S)* | 50 ± 4 μM              | ca. 50 μM                    | 5 μM               |
| Androst enol   | 52 ± 3 μM              | ca. 25 μM                    | 10 μM              |

* The indicated stereoisomers were used for predicting Ki values, whereas experiments were performed using mixtures of stereoisomers.

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

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