Dynamic Interaction between the Dual Specificity Phosphatase MKP7 and the JNK3 Scaffold Protein β-Arrestin 2*

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JNK scaffold proteins bind JNK and upstream kinases to activate subsets of JNK and localize activated JNK to specific subcellular sites. We previously demonstrated that the dual specificity phosphatases (DSPs) MKP7 and M3/6 bind the scaffold JNK-interacting protein-1 (JIP-1) and inactivate the bound subset of JNK (1). The G protein-coupled receptor (GPCR) adaptor β-arrestin 2 is also a JNK3 scaffold. It binds the upstream kinases ASK1 and MKK4 and couples stimulation of the angiotensin II receptor AT1aR to activation of a cytoplasmic pool of JNK3. Here we report that MKP7 also binds β-arrestin 2 via amino acids 394–443 of MKP7, the same region that interacts with JIP-1. This region of MKP7 interacts with β-arrestin 2 at a central region near the JNK binding domain. MKP7 dephosphorylates JNK3 bound to β-arrestin 2, either following activation by ASK1 overexpression or following AT1aR stimulation. Initial AT1aR stimulation causes a rapid (within 5 min) dissociation of MKP7 from β-arrestin 2. MKP7 then reassociates with β-arrestin 2 on endocytic vesicles 30–60 min after initial receptor stimulation. This dynamic interaction between phosphatase and scaffold permits signal transduction through a module that binds both positive and negative regulators.

Mitogen-activated protein kinases (MAPKs)1 are essential components of many signaling pathways, linking extracellular signals to changes in transcription factor activity and gene expression (2). The JNK MAPKs respond to cell stress, growth factors, cytokines, and hormones and are involved in a variety of responses, including apoptosis, embryonic development, cell growth, and the immune response (3). The MAPKKKs, MLK, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase (MEKK) families, activate the MAPKKs, MKK4, and MKK7, which in turn activate JNKs (3). Once activated, depending on the cellular environment, JNKs can phosphorylate transcription factors, including c-Jun and ATF2 (4). JNKs can also phosphorylate non-nuclear substrates, including the Bcl-2 family member BAD (5) and the 14-3-3 protein (6).

MAPKs are inactivated by Tyr phosphatases, Ser/Thr phosphatases, and DSPs/MKPs (7). The DSPs make up an evolutionarily conserved family that inactivates MAPKs by dephosphorylation of critical threonine and/or tyrosine residues (8, 9). DSPs, MKP7 (10, 11), M3/6 (hVH5), (12) and MKP5 (13, 14) specifically inactivate the JNKs. MKP5 null mice have shown that MKP5 is important for regulation of innate and adaptive immunity (15).

The response to particular stimuli and the subsequent cellular localization of active MAPKs is thought to be maintained by scaffold proteins (16). For example, the JIP family of scaffold proteins are kinesin cargo proteins that recruit MLKs, MKK7, and JNK (17, 18, 25) and also the DSPs, MKP7, and M3/6 (1). Such binding of both kinases and phosphatases has also been observed in the family of protein kinase A-anchoring proteins (AKAPs) (19); yotiao/AKAP450 binds both PKA and phosphatase PP-1 to control N-methyl-d-aspartate receptor signaling (20).

β-Arrestins are adaptors that control G protein-coupled receptor (GPCR) desensitization and internalization via clathrin-coated vesicles (21, 22); recently they have also been shown to act as MAPK scaffolds, recruiting signaling complexes to activated GPCRs. β-arrestin 2 binds JNK3 and upstream kinases ASK1 and MKK4 to stimulate JNK activation after triggering of the angiotensin type 1a receptor (AT1aR) (23, 24). In this report, we show that β-arrestin 2 also binds the DSP MKP7. After AT1aR stimulation, MKP7 transiently dissociates from β-arrestin 2 allowing transient JNK3 activation.

EXPERIMENTAL PROCEDURES

Plasmids—β-arrestin 2 was amplified by PCR using Turbo Pfu polymerase (Stratagene) from an expressed sequence tag (Integrated Molecular Analysis of Genomes and Their Expression Consortium clone number 3028154), and then full-length and all subsequent β-arrestin 2 deletions were subcloned into the pEBG vector and fused to glutathione S-transferase (GST). The MKP7 C terminus-only construct and M3/6 were subcloned into pCDNA4hisC and fused to an N-terminal Xp/P7 epitope tag. Xp-tagged MKP1, MKP2, hPAC1, MKP7, and MKP7 deletions (1) HA-JNK3 (1) and GST-JIP-1 (17) have been described previously. Plasmids expressing FLAG-JNK2, FLAG-β-arrestin 2, GFP-β-arrestin 2, and HA-ASK1 were a kind gift from Alan Whitmarsh (University of Manchester, UK), and the HA-AT1aR construct was a kind gift from Stephanie LaPorte (McGill University, Montreal, Canada).

Cell Culture—293T and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. 293T and COS-7 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

Pull-downs, Immunoprecipitations, and Immunoblots—293T cells were washed once with ice-cold buffered saline then lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 5 mM EDTA, and a protease inhibitor mixture (Roche Applied Science). After 10 min of incubation on ice, the extracts were centrifuged at 14000 × g for 15 min. Supernatants were subject to pull-down or im-

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAP kinase kinase; JNK, c-Jun NH2-terminal kinase; GPCR, G protein-coupled receptor; JIP, JNK-interacting protein; AT1aR, angiotensin type 1 receptor; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; DSP, dual specificity phosphatase.
FIG. 1. Selective binding of the dual specificity phosphatase MKP7 to MAPK scaffold β-arrestin 2. A, top panel, a plasmid expressing Xp-tagged MKP7 (0.75 µg) was introduced into 293T cells together with GST, GST-tagged β-arrestin 2, and GST-tagged JIP-1 (0.2 µg). GST-containing complexes were isolated with glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 in the precipitates was examined by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 4.5 and 1.2% of total expressed MKP7 was present in the GST-JIP-1 and GST-β-arrestin 2 precipitates, respectively. The relative expression levels of GST, GST-β-arrestin 2, GST-JIP-1, and MKP7 are also shown in the whole cell extract. Bottom panel, a construct expressing M3/6 (0.75 µg) was introduced into 293T cells...
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m noprecipitation, as described below. Samples were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Membranes were subjected to immunoblotting with anti-Xpress (Invitrogen), anti-M3/6 polyclonal (also used to detect GST), anti-FLAG M2 monoclonal (Sigma), or anti-RA high affinity (Roche) antibodies and horseradish peroxidase-conjugated secondary antibodies (Dako). Blots were developed using ECL reagents (Amersham Biosciences). For immunoprecipitation or pull-down experiments, extracts were made as described above and incubated with either glutathione-Sepharose 4B (Amersham Biosciences) for GST pull-down constructs or with anti-FLAG M2 with a GST protein G-Sepharose beads (Sigma) for 3 h. Beads were then washed three times in lysis buffer and resuspended in an appropriate amount of gel loading buffer (final concentration 50 mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 4% glycerol). For experiments to determine JNK phosphorylation, COS-7 cells were lysed directly into gel-loading buffer and blots probed with anti-phospho-JNK (Promega), anti-HA high affinity, anti-FLAG M2, or anti-Xpress antibodies.

Immunostaining—COS-7 cells were transfected on glass coverslips. The cells were incubated with 1 μM angiotensin II (Sigma) for the indicated times. The cells were then washed with phosphate-buffered saline solution, fixed with 4% paraformaldehyde (Sigma), and lysed with 0.2% Triton X-100 phosphate-buffered saline. Indirect immunofluorescence was performed by incubating the MKP7 polyclonal antibody with Alexa 568 conjugated secondary antibodies (Jackson ImmunoResearch) and was used and prepared on a Zeiss confocal microscope using Bio-Rad Lasersharp software.

RESULTS

Selective Binding of MKP7 to β-Arrestin 2—We recently reported that the DSPs, MKP7, and M3/6 bind the JNK scaffold proteins JIP-1 and JIP-2 (1). As β-arrestin 2 has been identified as a scaffold protein for JNK3, the neuronal isoform of JNK (23, 24), we asked whether any DSPs could bind β-arrestin 2. We co-expressed GST-tagged β-arrestin 2 with Xpress-tagged members of the DSP family and examined β-arrestin 2 precipitation for presence of the phosphatase using the anti-Xpress tag antibody. Under these conditions, MKP7 bound β-arrestin 2 (Fig. 1A, top panel, lane 2), whereas none of the other DSPs, including the highly related M3/6, was detected in the β-arrestin 2 precipitates (Fig. 1A, bottom panel, lane 2, and Fig. 1B, lanes 1, 2, 4, 6, and 8). We next performed co-precipitation analysis of MKP7 deletion mutants with β-arrestin 2 (Fig. 1, C and D). This identified a deletion mutant containing amino acids 1–394 that did not bind β-arrestin 2, whereas a mutant containing amino acids 1–443 did (Fig. 1D, lanes 6 and 8). The sequence between amino acids 394 and 443 is also critical for MKP7 binding to JIP-1. The equivalent region of M3/6 is very similar to that of MKP7, which suggests either that β-arrestin 2 (unlike JIP-1) discriminates between the two sequences or that M3/6 binding is not detected within the sensitivity of our assay. The MKP7 C-terminal fragment residues 360–665 were sufficient for binding both JIP-1 and β-arrestin 2, whereas residues 1–394 of MKP7 did not bind (Fig. 1E, compare lanes 5, 6, 8, and 9). As shown in Fig. 1F, MKP7-(1–394), which cannot bind β-arrestin 2, can still bind JNK3 (lane 3). Thus MKP7 binding to β-arrestin 2 is dependent on residues within its C-terminal region. This region is sufficient for the interaction, demonstrating that the interaction is independent of JNK3 binding to MKP7.

The Regions of β-Arrestin 2 That Interact with MKP7—We then performed co-precipitation analysis of β-arrestin 2 deletion constructs with MKP7. MKP7 bound weakly to the N-terminal region (1–164) and full-length β-arrestin 2 (Fig. 2B, (ii), lanes 5 and 7); however, MKP7 bound more strongly to a fragment of amino acids (165–410) of β-arrestin 2 (Fig. 2B, (i), lanes 2 and 3). To map the strong interaction site, further β-arrestin 2 constructs were analyzed. Residues between 195 and 202, previously identified as the JNK binding domain, were critical for strong binding (Fig. 2C, lanes 2 and 4, and Fig. 2D, lanes 4 and 6). To assess the role of JNK in this interaction, we examined the interaction between a β-arrestin 2 central fragment and the MKP7 C terminus, which does not bind JNK3. Amino acids 165–239 of β-arrestin 2 bound the MKP7 residues 360–665 (Fig. 2E, compare lane 5); this demonstrates an interaction between the MKP7 C terminus and the central JNK binding domain region of β-arrestin 2 that is independent of JNK3. The C terminus of MKP7 therefore interacts most strongly with a region between amino acids 195 and 202 of β-arrestin 2. Removal of the N-terminal region (1–164) of β-arrestin 2 increases MKP7 binding. It is possible that the N-terminal region may regulate MKP7 binding in the intact protein; for example a conformational change in β-arrestin 2 that displaces the N-terminal region might further enhance the strong binding region between amino acids 195 and 202.

MKP7 Dephosphorylates JNK Associated with β-Arrestin 2—β-Arrestin 2 binds JNK3 and its upstream kinases, ASK1 and indirectly MKK4, and enhances JNK3 phosphorylation (23, 24). We therefore assessed whether MKP7 could dephosphorylate β-arrestin 2-bound JNK, as we previously described for JIP-1-bound JNK (1). We overexpressed JNK3 with β-arrestin 2 and the MAP3K ASK1 in COS-7 cells and observed along with GST, GST-β-arrestin 2, and GST-JIP-1 (0.2 μg). GST-containing complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of M3/6 in the precipitates was examined by immunoblot using an anti-M3/6 polyclonal antibody. Densitometry analysis indicates 2.3% of total expressed M3/6 was present in the GST-JIP-1 precipitates. The relative expression levels of GST, GST-β-arrestin 2, GST-JIP-1, and M3/6 are also shown in the total extract. B, GST and GST-β-arrestin 2 (0.5 μg) were expressed in 293T cells along with Xp-tagged DSPs MKP1, MKP2, IP1C, and MKP7 (0.75 μg). β-arrestin 2-containing complexes were isolated with glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of the phosphatases in the GST precipitates was examined by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 1.0% of total expressed MKP7 was present in the GST-β-arrestin 2 precipitates. The expression levels of GST, GST-β-arrestin 2, and the phosphatases are also shown in the whole cell extract. C, the MKP7 deletion constructs used in these experiments. Numbers refer to the amino acid position. JIP DD, JIP docking domain; CDC25, CD25 homology domain; CD, DSP catalytic domain; NLS, nuclear localization sequence; NES, nuclear export sequence. D, constructs expressing GST or GST-β-arrestin 2 (0.2 μg) were introduced into 293T cells together with Xp-tagged MKP7 deletion constructs were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 in the GST precipitates was examined by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 0.90, 0.80, 0.76, and 0.82% of total expressed MKP7, MKP7-(1–443), MKP7-(1–522), and MKP7-(1–552), respectively, are present in the GST-β-arrestin 2 precipitates. Relative expression levels are also shown in the total extract. E, phospho-Xpress (0.5 μg) were expressed in 293T cells together with Xp-tagged MKP7-(1–394), MKP7-(360–665) construct (0.75 μg). GST complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of all MKP7 constructs was examined by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 3.8 and 1.3% of total expressed MKP7 was present in the GST-JIP-1 and GST-β-arrestin 2 precipitates, respectively. Similarly, 4.1 and 1.1% of total expressed MKP7-(360–665) was present in GST-JIP-1 and GST-β-arrestin 2 precipitates, respectively. The expression levels for GST and Xpress-tagged constructs were also examined using the appropriate antibodies. F, constructs expressing either control vector pCDNA3HisC or FLAG-tagged JNK3 (0.5 μg) were expressed in 293T cells along with Xp-tagged MKP7 or Xp-tagged MKP7-(1–364) (0.75 μg). FLAG-containing complexes were isolated by immunoprecipitation (IP) using the anti-FLAG M2 antibody conjugated to protein G from 5 × 10⁶ cells, and the presence of MKP7 constructs was examined by immunoblot using the anti-Xpress tag antibody. The expression levels for all constructs are also shown. Notice the heavy chain of the antibody is present in all lanes; however, in the presence of MKP7-(1–394), the band is much heavier, indicating the presence of the phosphatase in the immunoprecipitated complex.
FIG. 2. The interaction domains on β-arrestin 2 for MKP7. A, the β-arrestin 2 deletion constructs used in these experiments. Numbers refer to the amino acid positions. JBD, proposed JNK binding domain; P, GPCR phosphate sensor. Regions that bind MAPKKs and clathrin/AP2 are also indicated. B, (i) and (ii), plasmids containing GST, GST-β-arrestin 2 full-length, or deletion constructs 165–410 and 1–164 (0.2 μg) were...
enhancement of JNK activation as reported previously (23) (Fig. 3A, lanes 2 and 3). The addition of MKP7 inhibited the JNK3 activation stimulated by β-arrestin 2, whereas MKP7 did not affect phosphorylated JNK in the absence of scaffold (Fig. 3A, compare lanes 2 and 4; and lanes 3 and 5). This activity of MKP7 was dependent on its phosphatase activity, as a catalytically inactive mutant (1) did not affect JNK3 activation (Fig. 3B, lanes 3 and 5). These data show that MKP7 can inactivate the pool of JNK3 bound to β-arrestin 2.

Angiotensin II ligation to the AT1aR stimulates JNK3 phosphorylation in the presence of β-arrestin 2 (23). We co-expressed JNK3, β-arrestin 2, and AT1aR in COS-7 cells and showed that angiotensin II stimulated phosphorylation of JNK3 at 15 and 30 min, with the signal disappearing after 60 min (Fig. 3C, lanes 3 and 4), similar to previously published data (3). In the absence of β-arrestin 2 and addition of MKP7, stimulation of JNK3 phosphorylation remains the same as above (Fig. 3C, lanes 8 and 9). However, when MKP7 was expressed along with β-arrestin 2, JNK3 phosphorylation was equivalent at 15 min after angiotensin II stimulation but completely abrogated 30 min after stimulation (Fig. 3C, lanes 3 and 4; and 13 and 14). This data indicates MKP7 utilizes the interaction with β-arrestin 2 to specifically dephosphorylate JNK3, thus allowing a more transient JNK3 phosphorylation under AT1aR activation.

MKP7 Interacts with β-Arrestin 2 in Resting Cells but Is Released after AT1aR Stimulation—To explain the initial equivalent JNK3 activation seen in the presence of MKP7, followed by its rapid inactivation, we postulated that MKP7 interaction with β-arrestin 2 might be regulated by AT1aR stimulation. To detect MKP7 location, we used a previously described polyclonal antiserum (1) that detects a cytoplasmic domain. As MKP7 is rapidly inactivated, we postulated that MKP7 binds to JNK3, which results in JNK3 phosphorylation (15, 30), and ADP ribosylation factor 6 (31). In response to agonist binding, β-arrestin 2 also undergoes rapid ubiquitination by Mdm2, which is essential for receptor internalization (32).

In addition to their roles in receptor inhibition, β-arrestins are also signal transducers and have been shown to interact with intracellular signaling components, including both tyrosine and MAP kinases. For example, β-arrestins have been shown to recruit the Src family kinases (33) to endothelin ETA receptors (34), neurokinin 1 receptors (35), and CXCR1 receptors (36), resulting in the activation of the MAPK ERK1/2 (37). β-arrestin 2 has also been shown to directly recruit components of the ERK MAPK signaling module to the AT1aR (38). Although G protein-dependent ERK signaling is responsible for the activation of transcription factor Egr-1 after AT1aR stimulation, the β-arrestin 2-bound pool of ERK is retained in the cytoplasm, targeting activated ERK to other cytoplasmic sub-

To confirm this dynamic interaction between β-arrestin 2 and MKP7, we examined MKP7 binding to β-arrestin 2 after AT1aR stimulation. For these experiments, we used 293T cells to express sufficient protein for detection after immunoprecipitation rather than the COS-7 cells in which we measured subcellular localization and JNK3 dephosphorylation. In the absence of JNK3, the binding of MKP7 to β-arrestin 2 changed relatively little after angiotensin II stimulation (Fig. 4B, lanes 3–7). However with the addition of JNK3, MKP7 binding to β-arrestin was lost rapidly after angiotensin II stimulation, returning to the initial level after 60 min (Fig. 4B, lanes 9–13). The level of JNK3 binding to β-arrestin 2 remained the same throughout the time course, as previously described (23) (data not shown).

Together, these data demonstrate that activation of the AT1aR causes rapid dissociation of MKP7 from β-arrestin 2. This dissociation is dependent on the presence of JNK3 and is reversed 30–60 min after AT1aR stimulation. Because JNK3 is required for MKP7 dissociation from β-arrestin 2 triggered by angiotensin II, we asked whether JNK3 activation per se is sufficient to cause MKP7 dissociation. To do this, we used overexpressed ASK1 as a JNK3 activator. Fig. 4C shows that ASK1 causes a significant decrease in the amount of MKP7 bound to β-arrestin 2, suggesting that it is the stimulation of JNK3 phosphorylation that leads to MKP7 dissociation from β-arrestin 2.

**Discussion**

**β-arrestins** were first described as adaptor proteins involved in the desensitization and internalization of G protein-coupled receptors. After GPCR activation, the cytoplasmic tail of the receptor becomes phosphorylated by G protein-coupled receptor kinases, leading to β-arrestin recruitment. β-arrestin then blocks further G protein interaction and bind proteins required for the internalization of GPCRs, such as clathrin (26, 27), AP-2 (28), N-ethylmaleimide-sensitive factor (29), Ras-guanine nucleotide dissociation inhibitor stimulator (30), and ADP ribosylation factor 6 (31). In response to agonist binding, β-arrestin 2 also undergoes rapid ubiquitination by Mdm2, which is essential for receptor internalization (32).

**In addition to their roles in receptor inhibition, β-arrestins are also signal transducers and have been shown to interact with intracellular signaling components, including both tyrosine and MAP kinases.** For example, β-arrestins have been shown to recruit the Src family kinases (33) to endothelin ETA receptors (34), neurokinin 1 receptors (35), and CXCR1 receptors (36), resulting in the activation of the MAPK ERK1/2 (37). β-arrestin 2 has also been shown to directly recruit components of the ERK MAPK signaling module to the AT1aR (38). Although G protein-dependent ERK signaling is responsible for the activation of transcription factor Egr-1 after AT1aR stimulation, the β-arrestin 2-bound pool of ERK is retained in the cytoplasm, targeting activated ERK to other cytoplasmic sub-

expressed with Xp-tagged MKP7 (0.75 μg) in 293T cells. GST complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 was examined by immunoblot using the anti-Xpress tag antibody. In (i), densitometry analysis indicates 0.6 and 5.8% of total expressed MKP7 was present in GST-β-arrestin 2 and GST-(165–410) precipitates, respectively. In (ii), 0.5 and 2.3% of total expressed MKP7 was present in GST-β-arrestin 2 and GST-(1–164) precipitates, respectively. Relative expression levels are also shown. C, constructs containing GST or deletion constructs 165–190, 165–239, 165–278, and 165–363 (0.2 μg) were expressed together with Xp-tagged MKP7 (0.75 μg) in 293T cells. GST complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 identified by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 5.5, 4.3, and 5.7% of total expressed MKP7 was present in the GST-(165–239), GST-(165–278), and GST-(165–363) precipitates, respectively. Relative expression levels are also shown. D, GST, GST-β-arrestin 2, or deletion constructs 189–410 and 202–410 (0.2 μg) were expressed with Xp-tagged MKP7 (0.75 μg) in 293T cells. GST-containing complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 was examined by immunoblot using the anti-Xpress tag antibody. Densitometry analysis indicates 0.7, 6.0, and 1.1% of total expressed MKP7 was present in the GST-β-arrestin 2, GST-(189–410), and GST-(202–410) precipitates, respectively. Relative expression levels are also shown. E, GST, GST-β-arrestin 2, or deletion constructs 165–410, 189–410, and 165–239 (0.2 μg) were expressed with Xp-tagged MKP7-(360–665) (0.75 μg) in 293T cells. β-arrestin 2-containing complexes were isolated using glutathione-Sepharose beads (GST pull-down) from 5 × 10⁶ cells, and the presence of MKP7 C terminus was only examined by immunoblot using the anti-Xpress tag antibody. Relative expression levels are also shown.
FIG. 3. MKP7 dephosphorylates JNK associated with β-arrestin 2. A, plasmids containing HA-tagged ASK1 (0.1 μg), HA-tagged JNK3 (0.2 μg), FLAG-tagged β-arrestin 2 (0.1 μg), or Xp-tagged MKP7 (0.1 μg) were co-expressed in COS-7 cells. Whole cell extracts were made using 5 x 10⁸ cells and analyzed for the presence of phospho-JNK3 by immunoblot analysis. The presence of total JNK3, ASK1, β-arrestin 2, and MKP7 from the cell extracts were examined by immunoblotting with anti-HA-, anti-FLAG-, and anti-Xpress-tagged antibodies, respectively. The graph represents the level of JNK3 phosphorylation under each condition as arbitrary units assigned by a densitometer. B, constructs containing HA-tagged ASK1 (0.1 μg), HA-tagged JNK3 (0.2 μg), FLAG-tagged β-arrestin 2 (0.1 μg), or Xp-tagged MKP7 C-S mutant (0.1 μg) were co-expressed in COS-7 cells. Total cell extracts were made using 5 x 10⁸ cells and analyzed for the presence of phosphorylated-JNK3 by immunoblot using an antibody that recognizes the phosphorylated form of JNK. The presence of total JNK3, ASK1, β-arrestin 2 (β-ar2), and MKP7 C-S from the cell extracts were examined by immunoblotting with anti-HA tag and anti-FLAG tag antibodies and anti-MKP7 antiserum, respectively. The graph represents the level of JNK3 phosphorylation under each condition calculated by densitometry and showing levels as arbitrary units. C, Xp-tagged MKP7 (0.07 μg), HA-tagged JNK3 (0.1 μg), HA-tagged AT1aR (0.2 μg), and FLAG-tagged β-arrestin 2 (0.1 μg) were co-expressed in COS-7 cells. Cells were stimulated with 1 μM angiotensin II (Ang II) for the indicated time periods. Total cell extracts were made from 5 x 10⁸ cells and examined for the presence of phosphorylated JNK by immunoblot. The levels of JNK3, MKP7, and β-arrestin 2 were also examined using the appropriate antibodies (data not shown). The graph represents the level of JNK3 phosphorylation at each time point calculated by densitometry and showing levels as arbitrary units.
strates (39, 40). β-arrestin 2 was also recently reported to bind the neuronal JNK isoform, JNK3, and upstream components ASK1, and indirectly MKK4, and to stimulate JNK3 activation through AT1aR stimulation (23). Similar to ERK, JNK may bind β-arrestin 2 to maintain an extra nuclear location close to specific cytosolic substrates (41). As well as GPCRs, numerous receptors have been found that utilize β-arrestin-type scaffold proteins to coordinate both the kinases and phosphatases required for receptor function. These include the β-adrenergic receptors (42, 43), AT1R (44), and mGluRs (45).

We have shown that the dual specificity phosphatase MKP7 binds β-arrestin 2, using residues 394–443 of MKP7 previously

![Graph](Fig. 4. MKP7 transiently dissociates from β-arrestin 2 after AT1aR stimulation and JNK3 activation. A, plasmids expressing MKP7 (0.2 μg), GFP-tagged β-arrestin 2 (0.2 μg), HA-tagged JNK3 (0.2 μg), and HA-tagged AT1aR (0.2 μg) were co-expressed in COS-7 cells. After 48 h, the cells were stimulated with 1 μM angiotensin II (Ang II) for the indicated time periods. MKP7 was detected by immunofluorescence using an anti-MKP7 polyclonal antiserum with a Texas Red-conjugated donkey anti-rat secondary antibody. The images show the separate staining of MKP7 (red) and β-arrestin 2 (green), as well as a merge of the two; yellow indicates co-localization. Scale bars are included to indicate size. B, GST or GST-β-arrestin 2 (0.2 μg) were expressed together with HA-tagged AT1aR (0.4 μg) and either Xp-tagged MKP7 only (0.75 μg) or Xp-tagged MKP7 and HA-tagged JNK3 (0.75 μg). 293T cells were stimulated with 1 μM angiotensin II for the indicated times. GST-containing complexes were isolated using glutathione-Sepharose beads (GST pull-down), and the presence of MKP7 and JNK3 (data not shown) was examined by immunoblot. Relative expression levels are also shown. The graph represents the level of MKP7 binding to β-arrestin 2 at each time point calculated by densitometry and showing levels as arbitrary units. C, GST-β-arrestin 2 (0.2 μg) was expressed along with Xp-tagged MKP7 (0.75 μg) and HA-tagged JNK3 (0.75 μg) and/or HA-ASK1 (0.75–0.5 μg) in 293T cells. GST-containing complexes were obtained using glutathione-Sepharose beads (GST pull-down), and the presence of MKP7 was examined by immunoblot using the anti-Xpress tag antibody. Relative expression levels of all plasmids used are also shown. The graph represents the level of MKP7 binding to β-arrestin 2 in the presence of different concentrations of ASK1 calculated by densitometry and showing levels as arbitrary units.)
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identified as the JIP binding domain (1). The interaction of this region of MKP7 with these two diverse scaffold proteins suggests that it may function as a general scaffold-binding domain. The interaction between MKP7 and \( \alpha \)-arrestin 2 is dynamic, meaning they dissociate after \( \alpha \)-arrestin 2 is recruited to the AT1AR by angiotensin stimulation. Recent data have indicated \( \alpha \)-arrestin 2, upon activation by binding to the phosphorylated C terminus of GPCRs, undergoes a conformational change (46). This conformational change could be involved in the triggering of JNK3 activation. We have shown that the presence of JNK3 on the scaffold is necessary for MKP7 dissociation and also that ASK1 overexpression leads to MKP7 dissociation. We therefore propose that activation of JNK3 triggers MKP7 dissociation from \( \alpha \)-arrestin 2. After 30–60 min, depending on the cell system, MKP7 reassociates with \( \alpha \)-arrestin 2 to specifically dephosphorylate JNK3. This cycle of MKP7 dissociation from and reassociation with \( \alpha \)-arrestin 2 explains how a signal can be transmitted through a scaffold protein that apparently binds both activating and inhibitory components. By binding into this complex, under these circumstances, MKP7 may play a role in regulating JNK in the cytoplasm.

These studies have been performed in cells expressing the signaling components; however, endogenous interaction between \( \alpha \)-arrestin 2 and JNK3 has been seen in brain lysates (23), where the widely expressed MKP7 is also present (10, 11). Data has shown seven-transmembrane receptors Frizzled (47) and Smoothened (48, 49), as well as non-GPCRs, such as TGF\( \beta \) (50), are coupled to \( \alpha \)-arrestin scaffold proteins. It is therefore possible that MKP7 and other DSPs can regulate MAPK signaling in a number of receptor systems.

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