JNK3 Enzyme Binding to Arrestin-3 Differentially Affects the Recruitment of Upstream Mitogen-activated Protein (MAP) Kinase Kinases*

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Background: An interaction between arrestin-3 and MKK7 has never been elucidated.

Results: Arrestin-3 directly binds MKK7 and promotes JNK3α2 phosphorylation by MKK7 in vitro and in intact cells.

Conclusion: Arrestin-3 recruits JNK3α2 and both upstream MKKs.

Significance: Arrestin-3 promotes full JNK3α2 activation; MKK binding is regulated by JNK3α2.

Arrestin-3 was previously shown to bind JNK3α2, MKK4, and ASK1. However, full JNK3α2 activation requires phosphorylation by both MKK4 and MKK7. Using purified proteins we show that arrestin-3 directly interacts with MKK7 and promotes JNK3α2 phosphorylation by both MKK4 and MKK7 in vitro as well as in intact cells. The binding of JNK3α2 to arrestin promotes an arrestin-3 interaction with MKK4 while reducing its binding to MKK7. Interestingly, the arrestin-3 concentration optimal for scaffolding the MKK7-JNK3α2 module is ~10-fold higher than for the MKK4-JNK3α2 module. The data provide a mechanistic basis for arrestin-3-dependent activation of JNK3α2. The opposite effects of JNK3α2 on arrestin-3 interactions with MKK4 and MKK7 is the first demonstration that the kinase components in mammalian MAPK cascades regulate each other’s interactions with a scaffold protein. The results show how signaling outcomes can be affected by the relative expression of scaffolding proteins and components of signaling cascades that they assemble.

Arrestins specifically bind phosphorylated G-protein-coupled receptors, blocking further G protein activation, targeting receptors for internalization, and initiating alternative signaling (for review, see Refs. 1 and 2). Among four mammalian subtypes (3), two ubiquitously expressed non-visual arrestins, arrestin-22 and -3, act as multifunctional adaptors, interacting with dozens of non-receptors partners, including mitogen-activated protein (MAP)3 kinases (2, 4). MAP kinases form highly conserved signaling cascades stimulated by a wide variety of extracellular signals, such as growth factors, cytokines, and environmental stress (5). Canonical MAP kinase cascades consist of three kinases that successively phosphorylate and activate the downstream components (5, 6). In many cases signal propagation through these kinase cascades is regulated by scaffolding proteins that assemble the kinases into multiprotein complexes (7–9). Arrestins were reported to serve as scaffolds promoting the activation of all three main subfamilies of MAP kinases: JNK (10), ERK (11), and p38 (12).

The JNK signaling pathway is involved in the regulation of many cellular events, including growth control, transformation, and apoptosis (13). The JNK family consists of several ubiquitously expressed JNK1 and JNK2 isoforms and neuron-specific JNK3, which is also expressed in the heart and testes (14). Differential splicing and exon usage yield 10 different JNK isoforms (15). JNKs are activated by concomitant phosphorylation of a threonine and a tyrosine residue within a conserved Thr-Pro-Tyr (TPY) motif in the activation loop of the kinase domain. Interestingly, two upstream MAP kinase kinases, MKK4 and MKK7, preferentially phosphorylate distinct JNK activation sites: MKK4 phosphorylates tyrosine, whereas MKK7 phosphorylates threonine. The monophosphorylation of JNKs on either residue is reportedly sufficient to partially activate the kinase and induce phosphorylation of downstream substrates; however, full activation of JNKs requires both MKK4 and MKK7 (14, 16). Previous studies showed that biological functions of MKK4 and MKK7 are non-redundant despite the fact that they share several upstream activators and scaffold proteins (17). The actual mechanism is determined by different stimuli, physiological and pathological processes, and differential tissue distribution of MKKs (18). The biological significance and the underlying molecular mechanisms of differential activation of JNK kinases by distinct MKKs remain largely unknown.

Arrestin-3 was shown to bind ASK1, MKK4, and JNK3 and promote JNK3 activation (10, 19–22). Arrestin-3 scaffolding of the ASK1-MKK4-JNK3 cascade has been extensively studied (10, 19–23). However, whether arrestin-3 is able to promote JNK3 phosphorylation by MKK7 remains unknown. Here, we provide the first evidence that arrestin-3 scaffolds the MKK7-
JNK3α2 signaling complex. We found that the phosphorylation of the threonine residue within the TPY motif was promoted by arrestin-3 in COS-7 cells. We showed that arrestin-3 directly binds to MKK7, comparable to MKK4. Moreover, we found that JNK3α2 enhanced the association between arrestin-3 with MKK4 while reducing arrestin-3 binding to MKK7. We also compared the binding of arrestin-3 to active and inactive MKK4 and MKK7. In both cases active MKKs bind arrestin-3 as well as inactive ones, and JNK3α2 differentially regulates the binding of the two upstream MKKs to arrestin-3. This differential regulation of the binding of upstream kinases by JNK3α2 may underlie the higher optimal arrestin-3 concentration for JNK3α2 phosphorylation by MKK7 than by MKK4. We found that the two MKKs compete for arrestin-3 binding. Finally, we found that arrestin-3 facilitates JNK3α2 activation by either MKK in intact cells. These findings represent the first evidence that arrestin-3 is not only capable of scaffolding MKK4-JNK3α2 but also the MKK7-JNK3α2 signaling complex. This is also the first demonstration that the kinase components in mammalian mitogen-activated protein kinase (MAPK) cascades regulate each other’s interactions with scaffold proteins.

**EXPERIMENTAL PROCEDURES**

*Materials*—[γ-32P]ATP was from Perkin-Elmer (Waltham, MA). All restriction enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech (Manassas, VA) or Invitrogen. All other chemicals were from sources previously described (22).

*Protein Purification*—Arrestin-3, JNK3α2, GST-MKK4, and GST-tagged proteins were purified as previously described (22, 24).

**Purification of Tag-less MKK4—**GST-MKK4 was bound to glutathione–Sepharose high performance beads, which were re-suspended in 10 ml of a suspension buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.1% 2-mercaptoethanol, and 20% glycerol (pH 7.3). 20 units of thrombin (Novagen) were added to the suspension and incubated at room temperature overnight. The column was washed with ~3 column volumes of the same buffer and analyzed by SDS-PAGE. Tag-less MKK4 fractions were pooled and further purified by a Mono Q HR 10/10 anion exchange column that was eluted with 15–17 column volumes of Mono Q buffer containing 0.1 mM tosylphenylalanyl chloromethyl ketone) containing 0.5 mM NaCl, 0.2 mg/ml lysozyme, 1 mM MgCl2, and 20% glycerol. The mixture was incubated at 4 °C for 30 min, then Triton-100 was added to a final concentration of 1% (v/v), and the incubation at 4 °C was extended for another 30 min. The cell lysate was sonicated (at 5 s pulses with 5-s intervals) at 4 °C for 5 min and then cleared by centrifugation at 18,000 × g for 30 min. The supernatant was then agitated with nickel-nitritolactric acid beads for 1 h at 4 °C. After washing the beads with 200 ml of buffer A containing 10 mM imidazole and 20% glycerol, the GST-MKK7–His6 protein was eluted with 40 ml buffer B, pH 8.0, containing 200 mM imidazole and 20% glycerol. The eluted protein was collected and dialyzed into buffer S (25 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT) containing 20% glycerol. Protein concentration was measured using the Bradford reagent.

**Expression and Purification of Maltose-binding Protein (MBP)-tagged Arrestin-3**—To make MBP-arrestin-3, arrestin-3 cDNA was subcloned into pMal-p2T (25) (a generous gift from Dr. Noriyuki Matsuda, Tokyo Institute of Medical Science) between EcoRI and NotI sites. *Escherichia coli* strain BL21-codon plus (DE3)-RIL was used for expression. The cells were grown in LB at 30 °C overnight to A600 = 1.5–2.0 and then induced with 1 mM isopropylthio-D-galactoside and cultured for an additional 20 h at 25 °C with shaking at 250 rpm. The cells were then harvested, immediately flash-frozen in liquid nitrogen, and stored at −80 °C. Cells were then lysed in 20 ml of buffer A (20 mM Tris, pH 8.0, 0.03% Brij-30, 0.1% (v/v) β-mercaptoethanol, 5 mM imidazole, 1 mM benzamidine, 0.1 mM PMSF, and 0.1 mM tosylphenylalanil chloride) containing 0.5 mM NaCl, 0.2 mg/ml lysozyme, 1 mM MgCl2, and 20% glycerol. The mixture was incubated at 4 °C for 30 min, then Triton-100 was added to a final concentration of 1% (v/v), and the incubation at 4 °C was extended for another 30 min. The cell lysate was sonicated (at 5 s pulses with 5-s intervals) at 4 °C for 5 min and then cleared by centrifugation at 18,000 × g for 30 min. The supernatant was then agitated with nickel-nitritolactric acid beads for 1 h at 4 °C. After washing the beads with 200 ml of buffer A containing 10 mM imidazole and 20% glycerol, the GST-MKK7–His6 protein was eluted with 40 ml buffer B, pH 8.0, containing 200 mM imidazole and 20% glycerol. The eluted protein was collected and dialyzed into buffer S (25 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT) containing 20% glycerol. Protein concentration was measured using the Bradford reagent.

**Active MKK4 and MKK7—**GST-MKK4 (full-length wild-type human mitogen-activated protein kinase kinase 4 (MAP2K4) (GenBank™ accession number NM_003010) with an N-terminal cleavable GST tag) was expressed, purified, and activated essentially as described previously (21).

GST-MKK7–His6—4 μM GST-MKK7–His6 was activated by incubation with 2 μM GST-MKK1c (C-terminal 320 amino acids corresponding to the catalytic domain) (21) and 4 μM ATP for 60 min at 30 °C in 10 ml of activation buffer B (25 mM HEPES, pH 7.5, 2 mM dithiothreitol, 20 mM MgCl2, 0.1 mM EDTA, and 25 mM DTT) containing 50% glycerol. Protein concentration was measured using the Bradford reagent.
0.1 mM EGTA) and re-purified using a nickel affinity column that separates the active GST-MKK7-His<sub>6</sub> from GST-MEKK1c. The activated GST-MKK7-His<sub>6</sub> was stored in buffer S containing 20% glycerol at −80°C until further use.

**MBP Pulldown**—Binding of MKK4 and MKK7 to arrestin-3 was assayed by MBP pulldown with MBP-arrestin-3 immobilized on amylase resin (New England Biolabs) according to the manufacturer’s instructions. Briefly, 25 μl of purified MBP-arrestin-3 (5 μg) were incubated with 25 μl of amylase resin (50% slurry) in binding buffer (50 mM HEPES Na, pH 7.3, 150 mM NaCl) at 4°C with gentle rotation for 2 h. Subsequently, 100 μl of protein solutions containing active (phosphorylated by GST-MEKK1c) or inactive MKK4 or MKK7 (5 μg) along with different amounts of JNK3α2 (0, 2, 5, 10 μg) were added, and the suspensions were incubated at 4°C for 2 h. The suspension was transferred to centrifuge filters (Ultrafree, Millipore) and washed three times with binding buffer. The proteins were eluted from resin by the addition of 100 μl of elution buffer (50 mM maltose, 50 mM HEPES Na, pH 7.3, and 150 mM NaCl). Eluates were analyzed by SDS-PAGE and Western blotting. Samples obtained with MBP bound to the resin served as controls for nonspecific binding. To evaluate the effects of MKK7 on the arrestin3–M KK4 binding, the interaction between arrestin-3 and MKK4 was assayed using immobilized MBP-arrestin-3 (5 μg) to pull down tag-less MKK4 (5 μg) in the presence of various amounts of GST-MKK7 (0, 5, 10 μg). Eluates were analyzed by SDS-PAGE and Western blotting.

**GST Pulldown**—To test how many MKK molecules can bind arrestin-3 simultaneously, we performed GST pulldown using GST-MKK7 to pull down tag-less MKK4 in the presence of different amounts of arrestin-3. Briefly, 25 μg of GST-MKK7 (3 μg) was incubated with 25 μl of glutathione-agarose beads (50% slurry, Sigma) at 4°C for 2 h. Subsequently, 50 μl of protein solutions containing MKK4 (3 μg) and different amounts of arrestin-3 (0, 5, 10 μg) were added, and the suspensions were incubated at 4°C for 2 h. The suspension was transferred to centrifuge filters (Ultrafree, Millipore) and washed three times with binding buffer. The proteins were eluted from resin by the addition of 100 μl of elution buffer (50 mM reduced glutathione, 4 mM DTT, 50 mM HEPES Na, pH 7.3, 150 mM NaCl). Eluates were analyzed by SDS-PAGE and Western blotting.

**Cell Culture and Transient Transfection**—African green monkey cells (COS-7) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) plus penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. The cells were plated at 80–90% confluence and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were serum-starved overnight before all experiments and used 48 h post-transfection.

**Immunoprecipitation**—Cells (60-mm plates) were lysed in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 20 mM NaF, 1 mM sodium orthovanadate, 10 mM N-ethylemaleimide, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) for 60 min at 4 °C. After centrifugation (10 min at 9000 rpm, 4 °C), supernatants containing around 500 μg of total protein were pre cleared by incubation with 35 μl of protein G-agarose (50% slurry, Millipore) and washed three times with binding buffer. The proteins were eluted from resin by the addition of 0.2 mM ATP (4 °C). The reactions were stopped by the addition of 15 μl of SDS sample buffer and analyzed by Western blot with phospho-JNK3 (p-Tyr) or phospho-MKK4 (Thr-202/Tyr-204) antibodies, as indicated. 

**RESULTS**

**Arrestin-3 Promotes the Phosphorylation of Both Tyrosine and Threonine on JNK3α2**—Activation of JNK relies on two upstream MAP kinase kinases, MKK4 and MKK7, each of which preferentially phosphorylates a distinct site, tyrosine (MKK4) and threonine (MKK7) (Fig. 1B) (16, 17). Although to reach the maximal activity of JNK both tyrosine and threonine must be phosphorylated, JNK monophosphorylated at either site was reported to be capable of phosphorylating downstream substrates (16, 26). How JNK makes the appropriate choice of its upstream kinases is a challenging question, as the activation of JNK depends on a wide variety of factors, including the nature of a stimulus and relevant physiological and pathological
JNK3 Affects the Binding of MKK4 and MKK7 to Arrestin-3

![Diagram A](image1)

**Figure 2.** The phosphorylation of both tyrosine and threonine on JNK3α2 is enhanced by arrestin-3.

**A** shows the phosphorylation of both tyrosine and threonine on JNK3α2 by MKK4 and MKK7. **B** shows the phosphorylation of both tyrosine and threonine on JNK3α2 by MKK4 and MKK7. **C** shows the phosphorylation of both tyrosine and threonine on JNK3α2 by MKK4 and MKK7. **D** shows the phosphorylation of both tyrosine and threonine on JNK3α2 by MKK4 and MKK7.

Processes as well as differential tissue distribution of MKKs and scaffolding proteins. Previous studies showed that scaffold proteins play a significant role in determining the differential requirements of upstream kinases for JNK activation (27).

Although arrestin-3 was shown to scaffold the JNK3 signaling cascade, previous studies focused on the ASK1-MKK4-JNK3 module (10, 19–22). ASK1-initiated JNK3 activation apparently relied on the endogenous MKK4 and MKK7. To test whether the exogenous expression of JNK3α2, ASK1, or arrestin-3 influences endogenous MKK4 and MKK7 expression in cells, we quantified MKK4 and MKK7 using purified GST-MKK4 and GST-MKK7 proteins as standards (Fig. 2C). The results showed that the levels of endogenous MKK4 and MKK7 were not affected by the overexpression of ASK1, JNK3α2, and arrestin-3 individually or in different combinations, further supporting the notion that the expression of arrestin-3 underlies the observed increase in phospho-JNK3α2.

We used phospho-Tyr and phospho-Thr antibodies to determine whether arrestin-3 predominantly promotes monophosphorylation of the tyrosine site or full activation of JNK3α2. To avoid interference from other kinases, HA-JNK3α2 was immunoprecipitated with anti-HA high affinity antibody (rat, Roche Applied Science) (Fig. 2B). We found that co-expression of

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**Table:**

| **Means ± S.D.** | **p**-Value |
|------------------|------------|
| 0.01             | <= 0.001   |
| 0.001            | <= 0.001   |
| 0.0001           | <= 0.001   |

**Figure Captions:**

1. Western blots of JNK3α2 show effective phosphorylation of its substrate JNK3α2. Our data show that either MKK4 or MKK7 alone can only phosphorylate by MKK4 and MKK7, respectively. The presence of arrestin-3 does not alter the specificity of either MKK for its target (Fig. 1A).

2. To determine whether the phosphorylation of either site in JNK3α2 is enhanced by arrestin-3 in the cellular environment, we expressed ASK1, JNK3α2, and arrestin-3 individually and in different combinations in COS-7 cells. Co-expression of arrestin-3 with ASK1 + JNK3α2 increased the level of phospho-JNK3α2 ~10-fold (Fig. 2A), in agreement with previous reports (19–21). ASK1-initiated JNK3α2 activation apparently relied on the endogenous MKK4 and MKK7.

3. In COS-7 cells, the amounts of endogenous MKK7 (31.2 ± 10.1 fmol/10 μg of cell lysate protein) were ~8-fold higher than MKK4 (3.9 ± 0.3 fmol/10 μg of cell lysate protein) (Fig. 2C).

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arrestin-3 increased not only phospho-Tyr JNK3 but also phospho-Thr JNK3 (Fig. 2, B and D). The facilitation by arrestin-3 of threonine phosphorylation of JNK3 was a surprising finding, because MKK7 is the only MAP kinase kinase known to phosphorylate threonine residues on JNK3. To date no previous study using co-immunoprecipitation and mass spectrometry-based proteomics has reported an association between arrestin-3 and MKK7 (4, 10). These results demonstrate that arrestin-3 is able to promote JNK3 phosphorylation by MKK7, suggesting that the molecular mechanism of this phenomenon must be elucidated.

**JNK3 Binding Differentially Affects Direct Association of Arrestin-3 with MKK4 and MKK7**

Two distinct models can explain how arrestin-3 facilitates JNK3 activation by MKK7. It is conceivable that arrestin-3 directly associates with ASK1 (MAPKK kinase) and JNK3 (MAPK), whereas MKK7 (MAPKK) is recruited by ASK1 and JNK3 without directly associating with arrestin-3. It is also possible that arrestin-3 binds all three kinases directly to form a complete signaling module (Fig. 3A). Because co-immunoprecipitation data are inherently ambiguous, earlier studies interpreted essentially the same findings as evidence of indirect (10) or direct (19–21) arrestin-3 interaction with MKK4. Recently we definitively demonstrated direct interaction between MKK4 and arrestin-3 using two purified proteins (22). Therefore, we investigated the mechanism of arrestin-3-dependent JNK3 phosphorylation by MKK7 using the least ambiguous approach (Fig. 3). To this end, we used purified proteins (Fig. 3B) in the in vitro pulldown assays to compare the interactions of MKK7, MKK4, and MBP-arrestin-3, with equal amounts of MBP as negative controls (Fig. 3, C and D). We found that arrestin-3 directly binds both MKK4 and MKK7 with somewhat higher affinity for MKK4 than MKK7.
JNK3 Affects the Binding of MKK4 and MKK7 to Arrestin-3

No nonspecific binding to MBP was detected (Fig. 3C). These data provide the first experimental evidence that arrestin-3 directly binds MKK7.

It was reported previously that MKK4 binding to arrestin-3 was significantly enhanced in the presence of JNK3 (10, 19). These studies used co-immunoprecipitation from cell lysates, where the interaction could have been mediated and/or regulated by any of the hundreds of other cellular proteins. Therefore, it remained unclear whether the observed increases of arrestin-3 association with MKK4 were caused by JNK3α2 or by other proteins. Direct binding of arrestin-3 to MKK7 (Fig. 3) also raises the question as to whether JNK3α2 has any effect on the recruitment of MKK7 to arrestin-3. Therefore, we performed MBP pulldown of MKK4 and MKK7 in the absence or presence of varying amounts of purified JNK3α2 (0, 2, 5, 10 μg). We did not detect any nonspecific effects of JNK3α2 on MBP pulldown of GST-MKK7, GST-MKK4, or control GST (Fig. 3C). We found that JNK3α2 enhanced arrestin-3 binding to MKK4 (Fig. 3D), in agreement with previous reports (10, 19–21). Unexpectedly, the presence of increasing amounts of JNK3α2 progressively decreased arrestin-3 association with MKK7 (Fig. 3D). These data suggest that JNK3α2 binding differentially regulates the recruitment of MKK4 and MKK7 to arrestin-3.

All earlier measurements of the binding of arrestins with the kinases from the JNK3 cascade were performed with either inactive kinases or kinases expressed in intact cells. Thus, how the phosphorylation of these kinases affects their association with arrestin-3 remained unexplored. This question is particularly important in the case of MKK4 and MKK7 because the inactive forms serve as the substrates for ASK1, whereas the active forms are upstream kinases for JNK3. Therefore, we performed MBP pulldown to compare the binding of active and inactive MKK4 or MKK7 to arrestin-3. We found that both inactive and active MKKs bind arrestin-3 with comparable affinities (Fig. 4). Furthermore, we observed the same differential regulation by JNK3α2 of the interactions of arrestin-3 with active MKK4 and MKK7; JNK3α2 increased the binding of MKK4 but reduced the binding of MKK7 (Fig. 4). Thus, the phosphorylation of MKK4 and MKK7 does not significantly change their recruitment to arrestin-3.

Direct association of arrestin-3 with both upstream kinases raises a new question: whether MKK4 and MKK7 can bind arrestin-3 simultaneously or compete for the same or overlapping binding site(s)? To address this issue, we performed in vitro GST pulldown assays using immobilized GST-MKK7 (5 μg) as bait along with tag-free MKK4 (2 μg) in the presence of varying amounts of arrestin-3 (0, 5, 10 μg), with equal amounts of GST as negative control (Fig. 5A). If arrestin-3 associates with both MKK4 and MKK7 simultaneously, the presence of arrestin-3 could have increased the amounts of MKK4 retained by GST-MKK7 via the formation of an MKK4-arrestin-3-MKK7 complex. The results show that MKK4 was retained by GST-MKK7, which indicates that MKK4 and MKK7 can form hetero-oligomers. The addition of arrestin-3 decreased the amount of MKK4 retained by MKK7 (Fig. 5A). Thus, the MKK4-arrestin-3-MKK7 complex did not form under the conditions tested. These data suggest that arrestin-3 binding prevents the hetero-dimerization between MKK4 and MKK7.

Therefore, we tested whether MKK4 and MKK7 compete for arrestin-3 (Fig. 5B). We tested the binding of immobilized MBP-arrestin-3 (5 μg) and tag-free MKK4 (5 μg) in the presence of different amounts of GST-MKK7 (0, 5, 10 μg). The amount of MKK4 bound to MBP-arrestin-3 significantly decreased with the addition of MKK7 (Fig. 5B). These data indicate that MKK4 and MKK7 compete for arrestin-3, implying that the binding sites for the two MKKs likely overlap and suggesting that arrestin-3 is unlikely to bind both MKKs simultaneously.

Arrestins can assume several distinct conformations (28–33). The original hypothesis that different binding partners preferentially interact with arrestins in a particular conformation (34) was supported by the subsequent demonstration that ubiquitin ligases Mdm2 (35) and parkin (36) prefer “inactive” free arrestins, whereas ERK1/2 prefers a receptor-associated state (37). Interestingly, JNK3α2 was found to bind both equally well (35). The model where the binding of JNK3α2 induces a conformational change in the arrestin-3 molecule, which dif-
ferentially affects the binding of the two MKks, provides the simplest explanation of our data (Fig. 3). The ability of JNK3α2 to induce a conformational rearrangement in arrestin is consistent with the finding that it interacts with both arrestin domains (19). Characterization of the conformational changes in arrestin-3 induced by JNK3α2 is necessary to understand the structural basis of the differential regulation by JNK3α2 of the binding of MKK4 and MKK7. Regardless of the exact mechanism, these data are the first demonstration that kinases of the JNK3 cascade regulate each other’s interactions with a scaffold.

**Biphasic Effect of Arrestin Concentration on JNK3α2 Phosphorylation by MKK7**—Using purified proteins, we recently showed that arrestin-3 acts as a simple scaffold, facilitating JNK3 phosphorylation by MKK4 by bringing the two kinases together (22). To directly test how arrestin-3 promotes JNK3α2 phosphorylation by MKK7, we reconstituted arrestin-3-MKK4-MKK7-JNK3α2 signaling module from purified proteins (Fig. 6). We first tested whether arrestin-3 can be phosphorylated by either MKK7 or JNK3α2 and found that neither active MKK7 nor active JNK3α2 phosphorylated arrested-3 under our assay conditions (Fig. 6C). To evaluate the functional role of arrestin-3, we kept the concentrations of active MKK7 (50 nM) and its substrate unphosphorylated JNK3α2 (0.5 μM) constant and measured the extent of JNK3α2 phosphorylation in the presence of various arrestin-3 concentrations (0–45 μM). JNK3α2 phosphorylation by MKK7 demonstrated a biphasic dependence on arrestin-3 concentration, being enhanced by low and inhibited by higher concentrations (Fig. 6, D and E). Under these conditions, the optimal arrestin-3 concentration for the facilitation of JNK3α2 phosphorylation by MKK7 was ~5–8 μM, which is about 10 times higher than the optimal arrestin-3 concentration that promotes JNK3α2 phosphorylation by MKK4 (0.6 μM) (Fig. 6D, E; see also Ref. 22).

These results are consistent with our MBP pulldown data, which show direct interaction between arrestin-3 and MKK7 (Fig. 3). The higher optimal concentration of arrestin-3 necessary to promote JNK3α2 phosphorylation by MKK7 compared with MKK4 is consistent with the differential regulation of MKK4 and MKK7 binding by JNK3α2 (Fig. 3). Optimal concentrations of scaffold proteins are mainly determined by the binding affinities between a scaffold protein and its partners (38–40). Positive or negative cooperativity of the simultaneous binding of the two components to the scaffold protein may also contribute to affect the optimal concentration (39).

This biphasic dependence on scaffold concentration would be observed when the scaffold acts by directly binding both kinases and bringing them together without activating either MKK or its substrate (38–42). In the presence of scaffold, the activation of the downstream kinase (JNK3) is determined by the concentrations of the following complexes (where A stands for arrestin-3, J for JNK3, and M for MKK7): the complete scaf-
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**FIGURE 6. Biphasic effects of arrestin-3 on JNK3α2 activation by MKK7 and MKK4.**

A. a model showing the scaffolding mechanism of the two-kinase signaling module. A, J, and M stand for arrestin-3, JNK3α2, and upstream kinase MKK4 or MKK7, respectively. Kinases can exist in three different complexes: (a) directly interacting JM; (b) bound to the scaffold to form incomplete complexes containing a single kinase (JA or AM); (c) simultaneously tethered by a scaffold to form a complete two-kinase signaling complex JAM. Six affinity constants (K1–K6) describe the indicated binding equilibria. B, calculated concentrations of JM (dotted line, left y axis) and JAM (solid line, right y axis) complexes (KinTek Explorer 3.0; all six Kj values were set at 5 μM). C, active MKK7 and JNK3α2 do not phosphorylate arrestin-3. Purified arrestin-3 (1 μM) was incubated with active MKK7 (50 nM), active JNK3α2 (50 nM), or inactive JNK3α2 (0.5 μM) for 5 min. The reactions were stopped with SDS sample buffer, and the proteins were separated on 10% SDS-PAGE. The gel was dried and exposed to x-ray film. A representative autoradiogram from one of four experiments is shown. D, representative autoradiograms showing JNK3α2 phosphorylated by MKK4 (upper panel) and MKK7 (lower panel) at the indicated concentration of arrestin-3 (10-s incubation). The optimal arrestin-3 concentrations are indicated (*, for MKK4; **, for MKK7). E, the effect of arrestin-3 concentration on JNK3α2 phosphorylation by both MKK4 and MKK7 is biphasic. The bands from the autoradiogram gels were cut out, and the radioactivity was measured in a Tri-Carb liquid scintillation counter to quantify the incorporation of [32P]phosphate from [γ-32P]ATP into JNK3α2. Means ± S.D. (n = 3) are shown.

The experimentally measured arrestin-3 concentration optimal for facilitation of JNK3 phosphorylation by MKK7 (Fig. 6, D and E) is ~5 μM. This value is higher than the concentration of either JNK3α2 or MKK7 in these experiments, which appears counterintuitive. Therefore, we used the model shown in Fig. 6A (along with thermodynamic limitations inherent in this system of equilibria: K1 × K2 = K3 × K4 = K5 × K6) to test whether this is possible. To model the effect of arrestin-3 on JNK3 activation by MKK4 or MKK7, we calculated the predicted formation of two “meaningful” complexes that include JNK3 and its upstream kinase, JM and JAM, as a function of arrestin-3 concentration (Fig. 6B). The model requires the values of six equilibrium dissociation constants, K1 through K6 (Fig. 6A). Only the binding affinity between JNK3 and arrestin-3 was previously measured experimentally and found to be ~4–5 μM (22). To simplify the modeling, we set all constants at 5 μM. As could be expected, the model showed that the concentration of JM progressively decreased with an increasing concentration of arrestin-3 (Fig. 6B). The concentration of JAM demonstrated a biphasic dependence on the concentration of arrestin-3 and peaked at ~5.5 μM arrestin-3 (Fig. 6B). Thus, the modeling shows that the highest level of JAM can be achieved at a scaffold concentration exceeding that of either kinase.

The rates of enzymatic reactions are governed by factors that influence the steady-state levels of catalytic species; thus the effects of scaffold proteins on MAPK activation may be reflective of cooperativity in MAPKK-MAPK binding, which results in an increase in the steady-state concentration of an active scaffold complex. Thus, even though arrestin-3 binds MKK4 more tightly than it binds MKK7 (Fig. 3, C and D), the refer-
ence in the concentration of arrestin-3 that optimally promotes MKK4- and MKK7-dependent JNK3α2 phosphorylation may also reflect the differential effects of JNK3 on the binding of arrestin-3 to MKK4 and MKK7 (38, 40, 41). Our data suggest that JNK3α2 phosphorylation is characterized by a faster rate of the scaffolded complex JAM compared with the binary complex JM. Presumably, the formation of JAM occurs through JA or AM, and its steady-state level may be sensitive to the cooperativity of upstream and downstream kinase binding. Although an increase of JA or AM is expected to result in a higher steady-state level of the scaffolded complex JAM, which facilitates JNK3 activation, a further increase in scaffold concentration results in a higher ratio of JA and AM, which is predicted to inhibit JNK3 activation. Thus, the ratio of the concentrations of JA, AM, JAM, and JM is predicted to determine the output of phosphorylated JNK3. With positive cooperativity, a second component (e.g. MKK4) may prefer to bind the JA complex, inducing the formation of JAM at lower scaffold concentration. In contrast, with negative cooperativity (e.g. MKK7) a higher scaffold concentration may be required to reach the optimal steady-state level of JAM for highest output of p-JNK3.

**DISCUSSION**

Scaffolding proteins have emerged as critical elements in determining the specificity of intracellular signaling pathways (9). All evolutionarily conserved MAPK signaling pathways are organized by scaffold proteins (9, 43). MAPK scaffolds are extremely diverse, including kinase suppressor of Ras (KSR), JNK-interacting protein (JIP), paxillin involved in cell adhesion, MAPK kinase (MEKK1), etc. Non-visual arrestins were also reported to scaffold MAPK pathways leading to the activation of JNK3 (10, 19–21, 23), ERK1/2 (11, 37, 44), and p38 (12, 45). Most scaffolds are believed to use a simple tethering mechanism to facilitate the efficiency of signal transduction (38, 39, 42), although this model is rarely proved experimentally. Some scaffolds were shown to be directly involved in complex allosteric control of their binding partners. One well characterized example of these is yeast Ste5, which does not simply bring the kinases together, but also catalytically unlocks the Fus3 MAP kinase for activation (46, 47).

MAP kinases are fully activated when they are phosphorylated on both threonine and tyrosine in the activation loop (16). MKK4 and MKK7 are the upstream kinases for JNKs, and each preferentially phosphorylates a distinct site, tyrosine (MKK4) or threonine (MKK7). Importantly, the requirements for MKK4 and MKK7 for JNK activation are different, depending on the nature of the stimulus (18, 48). Targeted gene disruption in mice showed that simultaneous disruption of MKK4 and MKK7 genes was required to block JNK activation caused by environmental stress, whereas the disruption of the MKK7 gene alone was sufficient to prevent JNK activation caused by proinflammatory cytokines (49). The differential role of MKK4 and MKK7 in JNK activation can also be determined by distinct scaffold proteins (27). Previous studies performed in mouse embryonic fibroblasts showed that axin-mediated JNK activation depends mainly on MKK7 and Dvl-induced JNK activation almost equally depends on MKK4 and MKK7, whereas Epstein-Barr virus latent membrane protein-1-mediated JNK activation is primarily dependent on MKK4 (27). There can be various reasons for differential requirement of MKK4 and MKK7 for JNK activation, such as different expression levels of MKK4 and MKK7, scaffolds, and other regulators in particular cell types.
The mechanisms responsible for distinct roles of these two MKks in JNK activation by different stimuli remain largely unknown.

Arrestin-3 dependent JNK3 activation was discovered more than a decade ago (10), and subsequent studies revealed some mechanistic details of this process. Arrestin-3 scaffolding of the JNK3 signaling cascade was first reported to depend on arrestin-3 binding to G-protein-coupled receptors (10), whereas the later same group (23) and others (19–22) showed that free arrestin-3 is capable of performing this function. Moreover, structure-function analysis of arrestin-3 revealed that binding of all three kinases, ASK1, MKK4, and JNK3, is a function that can be separated from JNK3 (20) and that an arrestin-3 mutant that efficiently binds all three kinases but does not promote JNK3 phosphorylation can act as dominant-negative silent scaffold, suppressing JNK3 activity in the cell by recruiting it away from productive scaffolds (21). Our recent demonstration that arrestin-3-dependent facilitation of JNK3 phosphorylation by MKK4 can be reproduced with purified proteins in the absence of G-protein-coupled receptors (22) confirmed the idea that this is a receptor-independent process. This model is also supported by the finding that an arrestin-3 mutant with the deletion in the interdomain hinge, which greatly impairs its ability to bind receptors (50, 51), facilitated JNK3 activation as effectively as WT arrestin-3 (19, 21). Although all four mammalian arrestin subtypes bind the kinases in the ASK1-MKK4-JNK3 cascade (35, 52), only arrestin-3 facilitates JNK3 activation (10, 19, 20, 23). Our recent finding that arrestin-2 has much lower affinity for MKK4 than arrestin-3 (22) likely explains why arrestin-2 does not promote JNK3 phosphorylation even though it is expressed at 10–20-fold higher levels in most cell types (53, 54).

All previous studies focused on the ASK1-MKK4-JNK3 cascade. Full JNK activation requires the phosphorylation of both Tyr (by MKK4) and Thr (by MKK7) residues (16), but it remained unclear whether arrestin-3 can promote the phosphorylation of JNK3 by MKK7. Therefore, we used purified proteins to investigate the role of arrestin-3 in JNK3 phosphorylation by MKK4, MKK7, and the combination of MKK4 + MKK7. We found that arrestin-3 did not alter the sites of JNK3 phosphorylation by either MKK (Fig. 1). Using specific phospho-Tyr and phospho-Thr antibodies, we demonstrated that arrestin-3 facilitated the phosphorylation of both Tyr and Thr residues within the TPY motif of JNK3 in vitro (Fig. 1) and in COS-7 cells (Fig. 2). The enhancement of Thr phosphorylation by arrestin-3 demonstrates that it facilitates JNK3 phosphorylation by MKK7.

The binding of MKK4 to arrestin-3 is harder to detect than that of ASK1 or JNK3α2 (10, 19). The authors of the initial study found that although it was hard to detect the binding of MKK4 or MKK7 to arrestin-3 in cell lysates, MKK4 binding to arrestin-3 was significantly enhanced upon co-expression of ASK1 and JNK3 individually or together, whereas the interaction of MKK7 with arrestin-3 was still undetectable under the same conditions (10). Recently we demonstrated direct interaction of MKK4 with arrestin-3 using purified proteins (22). Here we extended our findings by comparing the binding of purified MKK4 and MKK7 to arrestin-3 and found that arrestin-3 binds both MKK7 and MKK4 directly (Fig. 3). We also evaluated the binding of active MKK4 and MKK7 to arrestin-3 and showed that the phosphorylation of MKK4 or MKK7 does not significantly affect their association with arrestin-3 (Fig. 4). Unexpectedly, we found that the binding of the downstream kinase JNK3α2 enhanced the association of arrestin-3 with MKK4 while reducing the binding of MKK7 (Fig. 3). JNK3 phosphorylation by either MKK4 or MKK7 demonstrated a biphasic dependence on arrestin-3 concentration (Fig. 6), which indicates that arrestin-3 functions as a scaffold bringing the two kinases together in both cases (38, 39). We also investigated whether both upstream MKKs bind arrestin-3 simultaneously or compete with each other. The data showed that MKK4 and MKK7 compete for arrestin-3 binding (Fig. 5), suggesting that these two MKKs engage the same or a significantly overlapping site(s) on arrestin-3. Demonstrated positive cooperativity of JNK3α2 and MKK4 binding to arrestin-3 (Fig. 3) may contribute to the relatively low optimal concentration of arrestin-3 in facilitating JNK3α2 phosphorylation by MKK4 (Fig. 6). In contrast, negative cooperativity of JNK3α2 and MKK7 binding to arrestin-3 (Fig. 3) may contribute to the ~10-fold higher optimal concentration of arrestin-3 to promote JNK3α2 phosphorylation by MKK7 (Fig. 6). Our findings are in agreement with theoretical models (38, 39). It was predicted that the concentration of a simple scaffold that directly binds two components and brings them together should biphasically affect signal transduction (38, 39), as our data demonstrate (Fig. 6). It was predicted that the scaffold concentration necessary for efficient signaling is determined by the binding affinities of the scaffold for its partners (38, 39). Our data (Fig. 6) suggest that positive or negative cooperativity between the proteins recruited by a scaffold may also determine the optimal scaffold concentrations (38, 39).

Scaffold proteins regulate the output by bringing signaling proteins into close proximity to each other, holding them in a particular orientation as well as by allosteric regulation of the enzyme and/or its substrate (9). Most scaffold proteins facilitate signal transduction simply by simultaneous recruitment of several components of the pathway. This tethering mechanism can significantly increase the effective local concentrations of enzymes and their substrates. However, there are functional limitations of this simple scaffolding mechanism, such as the inhibition of signaling by high concentrations of scaffold proteins (Fig. 6, D and E). To improve the efficiency, some scaffold proteins mediate the enzyme-substrate reactions through orientation and allosteric regulation. For example, to stimulate substrate ubiquitination effectively, a scaffold cullin-RING-F-box complex has to orient the substrate properly for the upstream E2 ubiquitin-conjugating enzyme (56, 57). Allosteric regulation was observed in the yeast mating system, where Ste5 scaffold allosterically “unlocks” its binding partner MAPK Fus3 to make it a good substrate for the MKK Ste7 (47). The cooperativity of the binding of JNK3α2 and both MKKs to arrestin-3 observed here (Fig. 3) appears to represent a more sophisticated regulatory mechanism (Fig. 6, D and E). Considering the lower levels of endogenous MKK4 (Fig. 2C), a positive effect of JNK3α2 on its binding could ensure that arrestin-3 recruits MKK4 more effectively than other binding partners, such as
MKK7 expressed at much higher level (Fig. 2C). We observed that relatively high arrestin-3 concentrations are optimal for JNK3 phosphorylation by this kinase (~5 μM, Fig. 6, D and E), potentially reflecting the negative cooperativity of binding. Modeling describing the formation of various complexes (Fig. 6A) predicts that at the arrestin-3 concentration optimal for the formation of the JAM complex, the concentration of the JM complex is reduced ~4-fold (Fig. 6B). Thus, the observed enhancement of JNK3α2 phosphorylation by arrestin-3 (Fig. 6, D and E) indicates that in the arrestin-3-MKK7-JNK3α2 complex the phosphorylation of JNK3α2 is significantly more efficient than in the MKK7-JNK3α2 complex. These results suggest that arrestin-3 regulates JNK3α2 phosphorylation by MKKs not just by tethering the two kinases together but potentially also by ensuring their optimal orientation relative to each other, which can account for a significant increase in the efficiency of the reaction between two proteins (58).

Qualitatively our data fit existing mathematical models remarkably well, but some quantitative aspects remain unexplained. Existing models (38–42, 59) do not take into account additional effects of scaffolds, particularly the fact that scaffolds impose a particular relative orientation of the two proteins that can increase reaction rates by orders of magnitude (58). In addition, scaffolds can “concentrate” reaction components; even low affinity binding to a scaffold would increase local concentration of both kinases in its vicinity, thereby making the formation of JAM complexes more likely than when the kinases are evenly distributed in the solution. Our data suggest that more sophisticated mathematical models of scaffolded signaling cascades need to be developed. The positive effect of JNK3α2 binding on MKK4 recruitment is biologically relevant; it likely helps MKK4 expressed at low levels (Fig. 2) to compete with other arrestin-3 binding partners, including higher expressed MKK7.

Arrestin-3 was shown to bind dozens of different partners (4), whereas the size of arrestin-3 suggests that it can accommodate no more than 3–4 proteins simultaneously (1). To perform their functions, non-visual arrestins are often required to assemble particular combinations of proteins, such as three kinases belonging to the same MAP pathway, to form a productive signaling complex. How exactly arrestins manage to recruit proper combinations of partners remains largely unknown. Mechanistically, the change in arrestin conformation upon binding of certain partners could regulate the association of others. The best characterized example of this type of regulation is arrestin association with the internalization machinery; G-protein-coupled receptor binding induces the release of the arrestin C-tail (29–32), exposing clathrin (60) and AP2 (61) binding sites localized on this element. Arrestins exist in several distinct conformations: free, revealed by most crystal structures (22, 62–66), receptor-bound (29, 30), and microtubule-bound (28, 51). Recent structures of truncated “preactivated” (67) arrestin-2 in complex with a multiphosphorylated C-terminal peptide of vasopressin V2 receptor and a nanobody (68) and of a short splice variant of arrestin-1 (69) likely represent an intermediate state between free and receptor-bound conformations. Several arrestin binding partners show distinct preference for particular conformations; ERK1/2 prefers receptor-bound arrestins (37), whereas ubiquitin ligases Mdm2 and parkin preferentially interact with arrestins in the basal conformation (35, 36). Although we showed unambiguous evidence that free arrestin-3 binds MKK4, MKK7, and JNK3α2 and facilitates JNK3α2 phosphorylation, the role of the receptor in arrestin-mediated JNK3α2 activation remains unclear. The most straightforward approach to elucidation of the role of G-protein-coupled receptors in this process is the use of direct biochemical and biophysical methods to study arrestin-3-MAPK complexes reconstructed with purified proteins in the presence and absence of receptors under carefully controlled conditions.

Human cells express >20 different MAPKK kinases (MAPKKK), 7 MAPKK, and 11 MAPKs (70), forming an amazing variety of signaling modules with distinct cellular functions (9, 59, 71). Scaffolding proteins play critical roles in keeping signals accurately and specifically transmitted through these pathways. Differential regulation of the binding of arrestin-3 to MKK4 and MKK7 by JNK3α2 (Fig. 3) demonstrates one possible mechanism of how a scaffold selects proper binding partners upon recruiting another component in the same pathway. Our data (Fig. 6) support theoretical models (38, 39) suggesting that this process is regulated by relative concentrations of scaffolds and kinases involved. Although the JNK3α2-arrestin-3 complex prefers MKK4 (Fig. 3), under appropriate conditions arrestin-3 facilitates JNK3α2 activation by MKK7 as well (Figs. 6 and 7). These findings support the idea that differential requirements for MKK4 and MKK7 for JNK3α2 activation depend not only on the type of stimulus (59) but also on the expression levels of relevant scaffolding proteins. These data are the first direct experimental evidence that components of mammalian MAPK cascades can regulate the binding of each other to scaffold proteins. Our data also suggest that in rod photoreceptors, where arrestin-1 is expressed at very high levels, reaching up to 2 mM concentration in the cell body in the dark (72–74), its reported interactions with JNK3α2 (35) and upstream kinases (55) is likely to suppress the signaling in this pathway rather than enhance it.

In conclusion, we demonstrated that arrestin-3 promotes JNK3 phosphorylation by MKK7. The data suggest that although arrestin-3 binds MKK7 with only somewhat lower affinity than MKK4, JNK3 binding has the opposite effect on arrestin-3 interactions with MKK4 and MKK7, enhancing the former and strongly suppressing the latter. This differential regulation suggests that arrestin-3 might undergo a significant conformational change upon the binding of JNK3α2. Further experimentation with pure proteins to explore this conformational change is necessary. To better understand the scaffolding function of arrestin-3, the effects of other components (ASK1, receptor) need to be carefully explored as well. It will be important to test whether other scaffold proteins in MAPK pathways are regulated in a similar way to maintain the accuracy and specificity of signal transduction.

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