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How Does Arrestin Assemble MAPKs into a Signaling Complex?*

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Arrestins bind active phosphorylated G protein-coupled receptors, precluding G protein activation and channeling signaling to alternative pathways. Arrestins also function as mitogen-activated protein kinase (MAPK) scaffolds, bringing together three components of MAPK signaling modules. Here we have demonstrated that all four vertebrate arrestins interact with JNK3, MKK4, and ASK1, but only arrestin3 facilitates JNK3 activation. Thus, the functional specificity of arrestins is not determined by differential binding of the kinases. Using receptor binding-impaired mutant, we have shown that free arrestin3 readily promotes JNK3 phosphorylation. We identified key arrestin-binding elements in JNK3 and ASK1 and investigated the molecular interactions of arrestin2 and arrestin3 and their individual domains with the components of the two MAPK cascades, ASK1-MKK4-JNK3 and c-Raf-1-MEK1-ERK2. We found that both arrestin domains interact with all six kinases. These findings shed new light on the mechanism of arrestin-mediated MAPK activation and the spatial arrangement of the three kinases on arrestin molecule.

Arrestins are multifunctional regulators of cell signaling (1, 2). Arrestins, which bind active phosphorylated G protein-coupled receptors (GPCRs),[2] which play a major role in receptor desensitization and internalization (3, 4). With the identification of numerous non-receptor binding partners, the classical paradigm of arrestin function has been expanded, implicating arrestins in mitogen-activated protein kinase (MAPK) activation, protein ubiquitination, chemotaxis, apoptosis, and other cellular functions (2, 5–11).

The first indication that arrestins function as signaling adapters came from the studies of arrestin-dependent c-Src recruitment to the receptors, which results in the activation of extracellular signal-regulated kinases (ERK1/2) (10, 12, 13). Subsequently, arrestin2 and arrestin3 in complex with different receptors were reported to scaffold JNK3 (9), ERK1/2 (8, 14), and p38 (15, 16) activation cascades. Although arrestins play an important role in regulating different MAPK pathways, the mechanism of arrestin-dependent assembly of MAP kinases into a signaling complex remains largely unexplored. Existing models have limited predictive value. For example, the idea that JNK3 is activated solely by arrestin3 because this arrestin subtype has unique ability to bind JNK3 (9, 17) was not supported by further experimentation (18–20). Similarly, the hypothesis that only receptor-bound arrestins interact with MAP kinases (8, 9) was not confirmed (17–20).

Here we addressed several key mechanistic issues in arrestin-dependent MAPK signaling. First, we show that the scaffolding function is not limited to receptor-bound arrestin; free arrestin3 facilitates ASK1-mediated JNK3 activation, indicating that arrestins are not exclusively receptor-regulated adapters as thought previously. Second, we show that all four mammalian arrestins bind each component of the JNK3 cascade with comparable affinity, demonstrating that binding does not necessarily translate into activation. This finding establishes the mechanistic basis of the "dominant-negative" effect of certain arrestin subtypes. Third, using truncated forms of ASK1 and JNK3, we identified the major arrestin-binding elements of these two kinases. Finally, we show that every kinase in JNK3 and ERK2 activation cascades binds both arrestin domains. Based on these findings, we propose a functional model of arrestin-dependent regulation of MAPK activity and a new structural model of the arrestin-MAPK multiprotein signaling complex.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The coding sequences of bovine arrestin1 (rod), arrestin2, arrestin3, and human arrestin4 (cone)[3] with the C-terminal FLAG tag were subcloned into pcDNA3. Arrestin2 with engineered nuclear export signal (NES) (Q394L), NES-less arrestin3 (L393Q), and "inactive" (D7) mutants with a 7-residue deletion in the interdomain hinge were described previously (19, 21). Separated N and C domains of arrestin3 (residues 1–181 and 180–408) and arrestin2 (residues 1–180 and 179–418) were engineered with C-terminal NES and FLAG tag. All constructs were verified by dideoxy sequencing. Expression constructs for GFP-JNK3, HA-JNK3, and HA-MKK4 were gifts from Drs. Louis Luttrell (Medical University of South Carolina), Robert J. Lefkowitz (Duke Uni-

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; ASK, apoptosis signal-regulating kinase; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK, MAPK/ERK kinase; NES, nuclear export signal; NLS, nuclear localization signal; HEK, human embryonic kidney; PBS, phosphate-buffered saline; WT, wild type.

[3] Note that here we have used the systematic names of arrestin proteins: arrestin1 (also known as visual or rod arrestin, 48-kDa protein, or S-antigen), arrestin2 (β-arrestin or β-arrestin1), arrestin3 (β-arrestin2), and arrestin4 (cone or X-arrestin).

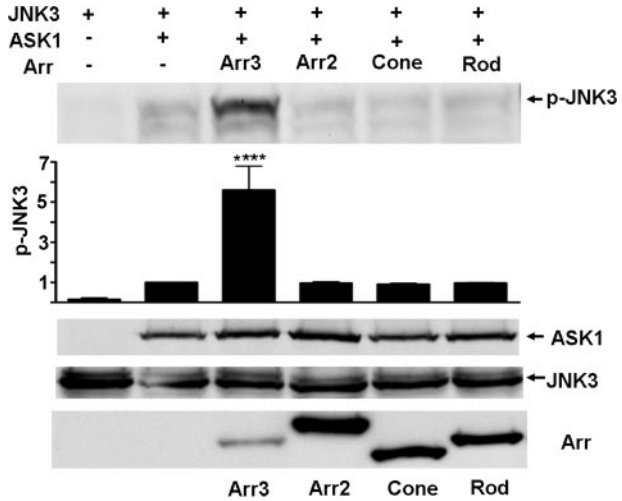

FIGURE 1. **Arrestin3 is the only isoform that enhances ASK1-mediated JNK3 activation.** COS-7 cells were transfected with HA-JNK3 alone, or with HA-ASK1, or together with FLAG-tagged arrestin3 (Arr3), arrestin2 (Arr2), arrestin1 (Rod), or arrestin4 (Cone). Cell lysates were immunoblotted for phospho-JNK (p-JNK3), HA, or FLAG. The intensity of the phospho-JNK band in three independent experiments was quantified and statistically analyzed. Means ± S.D. are shown. ****, $p < 0.0001$, as compared with the basal level in cells co-expressing ASK1 and JNK3 without arrestin.

versity), and Jia Le Dai (The University of Texas M. D. Anderson Cancer Center), respectively. HA-ASK was modified by oligo 5′-CCG AAG AAA AAG CGC AAG GTC-3′ to introduce a nuclear localization signal (NLS), and GFP-JNK3 was modified by deleting 39 N-terminal residues, 20 C-terminal residues, or both termini.

Cell Culture and Transient Transfection—Adenovirus-transformed human embryonic kidney cells (HEK-293A) and COS-7 African green monkey cells 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_2. 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.

Nuclear Exclusion Assay—The day after transfection HEK-293A cells were seeded onto Lab-Tek CC2 chambered slides coated with fibronectin (20 μg/ml in PBS) (Sigma) for microscopy and onto poly-D-lysine (15 μg/ml)-coated 24-well plates for Western blot. For microscopy cells were fixed in 4% paraformaldehyde on ice (15 min), permeabilized with 0.1% Triton in PBS, and blocked with 3% bovine serum albumin in PBS for 1 h at room temperature. FLAG-tagged arrestins were visualized with M2 anti-FLAG antibody (Sigma) followed by the Alexa 593 (red) anti-mouse secondary antibodies (Molecular Probes, Eugene, OR). GFP-JNK3 was visualized by its intrinsic green fluorescence, using an epifluorescence microscope equipped with a charge-coupled device camera. The slides were air-dried and mounted in the medium containing 4,6-diamidino-2-phenylindole to visualize the nuclei. The images were acquired using a Nikon EC2000 inverted fluorescent microscope. The distribution of full-length and truncated JNK3, ASK1-NLS, and the indicated arrestin in at least 20 cells was scored based on the subcellular distribution of the green fluorescence signal (nucleus > cytoplasm; nucleus = cytoplasm; nucleus < cytoplasm).

Western Blot—COS-7 cells were incubated with phosphatase inhibitors (50 mM NaF, 10 mM Na_3VO_4) in serum-free medium for 15 min at 37 °C, washed with cold PBS, and lysed with SDS sample buffer containing 10 mM NaF, 100 μM Na_3VO_4, 2 mM EDTA, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Whole cell lysates were boiled

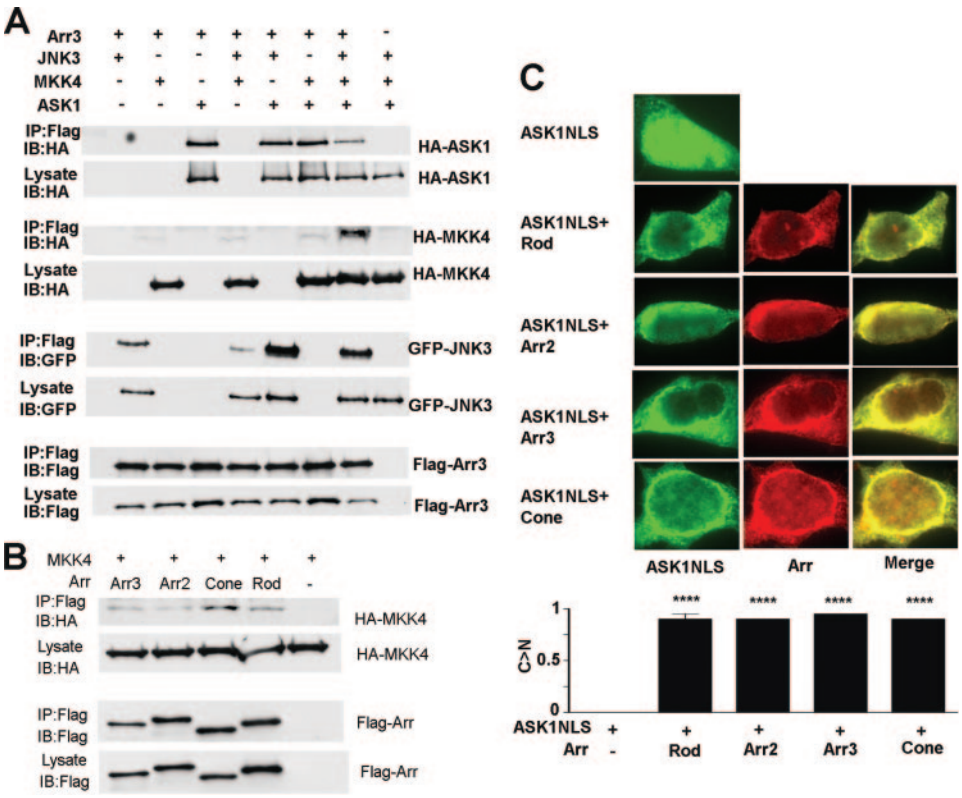

FIGURE 2. **ASK1, MKK4, and JNK3 bind arrestin3.** *A*, COS-7 cells were transfected with FLAG-arrestin3 (FLAG-Arr3) with GFP-JNK3, HA-ASK1, HA-MKK4, or with indicated combinations of these kinases. Cell lysates were immunoprecipitated with rabbit anti-FLAG antibody, and then immunoblotted with mouse anti-HA, anti-GFP and anti-FLAG antibodies. *B*, COS-7 cells were transfected with HA-MKK4 with indicated FLAG-tagged arrestins. Cell lysates were immunoprecipitated and immunoblotted as in panel A. *C*, HEK-293A cells were transfected with HA-ASK-NLS alone or with indicated FLAG-tagged arrestins. Arrestins were visualized with M2 anti-FLAG, HA-ASK1 with rat anti-HA high affinity antibody, followed by Alexa 593 anti-mouse (Red) and Alexa 488 anti-rat (Green) secondary antibodies, respectively. The representative images show arrestins (*red*), HA-ASK1-NLS (*green*), and both channels merged. At least 20 cells expressing indicated proteins were scored for the subcellular distribution of HA-ASK1-NLS. Means ± S.D. ($n = 3$) of the fraction of cells with more ASK1 in the cytoplasm than in the nucleus are shown. ****, $p < 0.0001$.

for 5 min and then centrifuged at 10,000 × *g* for 10 min, and the supernatants were used for Western blot. The proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Mouse monoclonal antibodies against FLAG (Sigma), HA (Sigma), GFP (Clontech), and phospho-JNK (Cell Signaling Technology Inc.) were used at 1:1000 or 1:2000 dilution followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. Protein bands were detected by enhanced chemiluminescence (ECL, Pierce) followed by exposure to x-ray film. Immunoblots were quantified using QuantityOne software (Bio-Rad Laboratories).

Immunoprecipitation—Cells (60-mm plates) were lysed in 0.75 ml of lysis buffer (50 mM Tris, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 20 mM NaF, 1 mM Na orthovanadate, 10 mM *N*-ethylmaleimide, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) for 30–60 min at 4 °C. In experiments involving ERK2, prior to lysis the cells were treated with 1 mM cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Pierce) for 30 min followed by 2 mM Tris-HCl, pH 7.5, for 15 min at room temperature. After centrifugation, supernatants were precleared by 20 μl of protein G-agarose. Then, 600 μl of supernatant was incubated with primary antibodies for 2 h followed by the addition of 12 μl of protein G-agarose beads for 2 h or overnight. The beads were washed three times with 1 ml of lysis buffer, and the proteins were eluted with 50 μl of sample buffer, boiled for 5 min, and analyzed by Western blot as described above.

Statistical Analysis—Quantitative data from at least three experiments were analyzed by one-way analysis of variance with arrestin as a main factor (with Bonferroni-Dunn correction for multiple comparisons).

RESULTS

Interaction Does Not Always Mean Activation, as All Four Mammalian Arrestins Bind ASK1, MKK4, and JNK3, but Only Arrestin3 Enhances JNK3 Phosphorylation—Mammals have four arrestin isoforms (nonvisual arrestin2 and arrestin3, arrestin1 (expressed in rods and cones), and arrestin4 (cone-specific) (1, 22)), which share a high degree of structural homology (23). Previously we showed that all four arrestins bind JNK3 (19, 20). However, we found that arrestin3 is the only isoform that enhances JNK3α2 phosphorylation in COS-7 cells (Fig. 1), in agreement with previous reports (9, 17). Because JNK3 activation requires the assembly of all three components of the cascade, we tested whether other arrestins bind upstream kinases ASK1 and MKK4. Arrestin interaction with ASK1 was tested in a nuclear exclusion assay based on the ability of arrestins equipped with the NES to remove their interaction partners from the nucleus (19, 20). The addition of a short C-terminal NLS, KKKRK, was sufficient to ensure nuclear localization of ASK1 (Fig. 2*C*). Co-expression of each of the four arrestins relocalized ASK1-NLS to the cytoplasm, demonstrating that all isoforms bind ASK1 (Fig. 2*C*). Similarly, we found that all four arrestins co-immunoprecipitate MKK4 (Fig. 2*B*). Among the three members of the ASK1-MKK4-JNK3 cascade the interaction with MKK4 appears to have the lowest affinity; it was detectable only at higher levels of MKK4 expression. However,

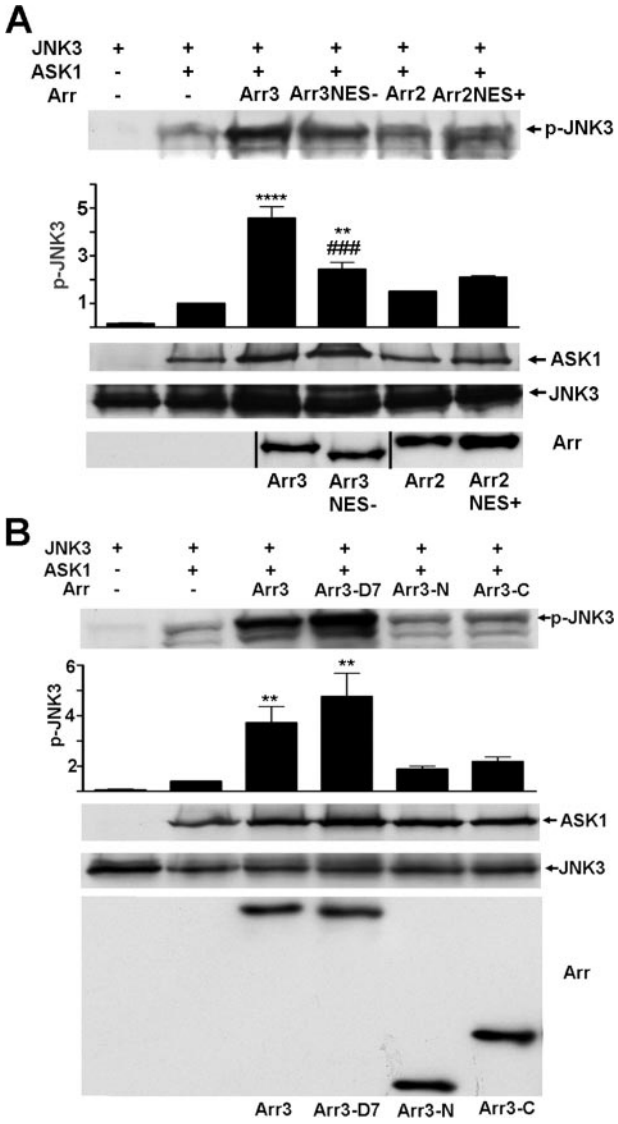

FIGURE 3. **Free arrestin scaffolds ASK1-MKK4-JNK3 module.** *A*, COS-7 cells were transfected with HA-JNK3 alone, or with HA-ASK1, or together with arrestin3 (Arr3), arrestin3-NES- (Arr3-NES-), arrestin2 (Arr2), or arrestin2-NES+ (Arr2-NES+). Cell lysates were analyzed as in Fig. 1 (untagged arrestin3 was detected with F4C1 mouse monoclonal antibody). Means ± S.D. ($n = 3$) of the intensity of phospho-JNK3 band are shown. Both arrestin3 (****, $p < 0.0001$) and arrestin3-NES- (**, $p < 0.01$) enhance JNK3 activation, as compared with cells expressing ASK1+JNK3 without arrestin; JNK3 activation enhanced by arrestin3-NES- was significantly different from arrestin3 (###, $p = 0.0004$). *B*, COS-7 cells were transfected with HA-JNK3 + HA-ASK1, or together with FLAG-tagged arrestin3, arrestin3-D7, arrestin3-N-domain, or arrestin3-C-domain. The lysates were analyzed as in Fig. 1. Means ± S.D. ($n = 3$) of the intensity of phospho-JNK3 band are shown. Statistical analysis shows that arrestin3 (**, $p = 0.0053$) and arrestin3-D7 (**, $p = 0.0018$) enhance JNK3 activation, whereas arrestin3-N ($p = 0.5769$) and arrestin3-C ($p = 0.3629$) do not.

both nonvisual arrestins bind MKK4 similarly, whereas arrestins 1 and 4 demonstrate higher binding (Fig. 2*B*). Thus, all members of the arrestin family interact with each kinase in the ASK1-MKK4-JNK3 cascade, so that the binding *per se* does not explain the unique ability of arrestin3 to facilitate JNK3 activation.

Because arrestin3, in contrast to arrestin2, has leucine-rich NES promoting its transport to the cytoplasm (18, 24), we tested whether the presence of NES affects arrestin-dependent JNK3 activation. All four mammalian arrestins are predomi-

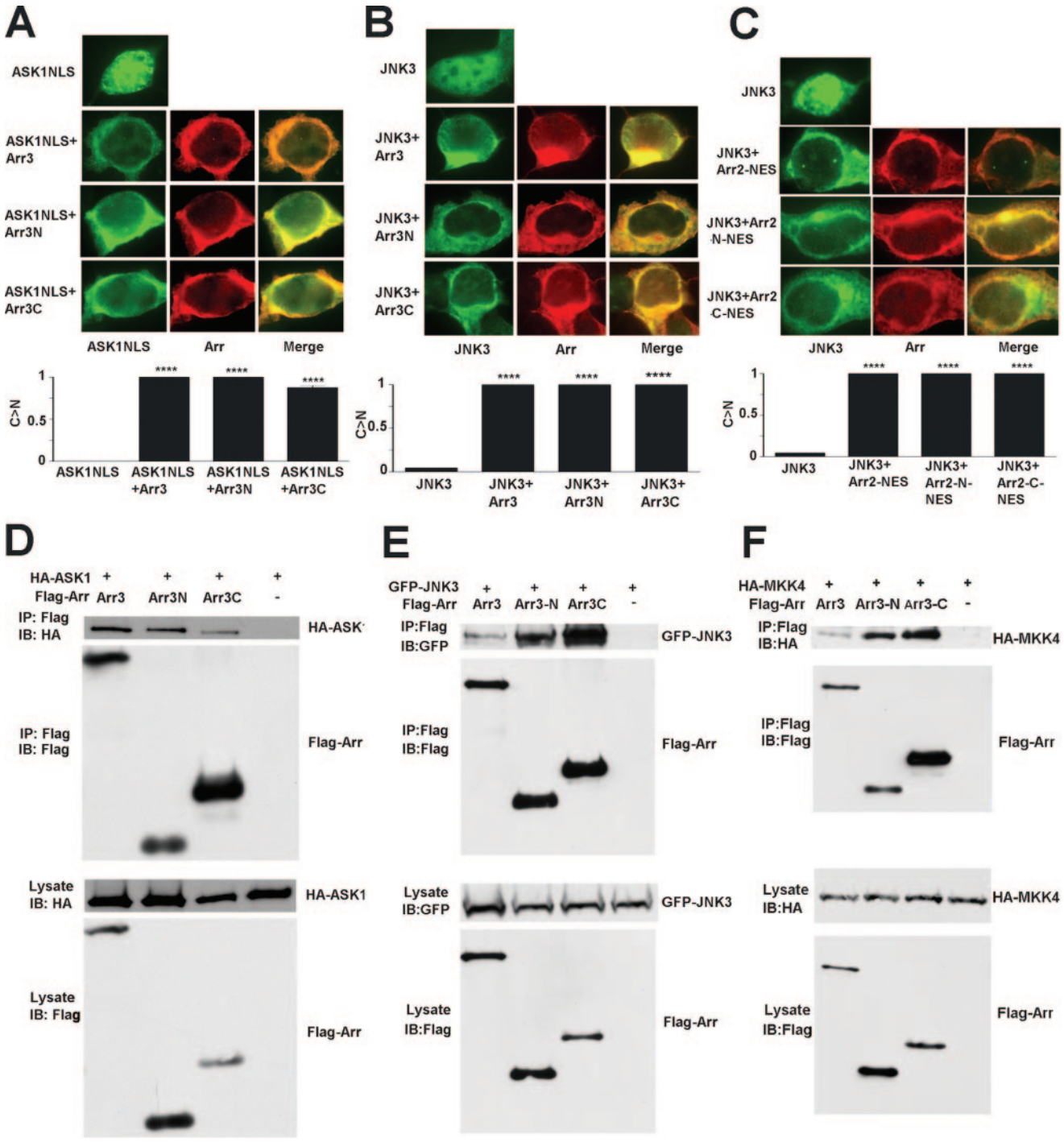

FIGURE 4. **ASK1, MKK4, and JNK3 interact with both domains of arrestin.** *A–C*, HEK-293A cells were transfected with HA-ASK-NLS or GFP-JNK3 individually or with FLAG-tagged full-length arrestin3 (FLAG-Arr3), its N- (FLAG-Arr3-N) or C-domain (FLAG-Arr3-C), arrestin2-NES+ (Arr2-NES), arrestin2 N-domain-NES (Arr2-N-NES), or arrestin2 C-domain-NES (Arr2-C-NES). Immunocytochemistry was done as described in Fig. 2*C*. GFP-JNK3 was visualized by its intrinsic fluorescence. Representative images are shown. The localization of GFP-JNK and HA-ASK1-NLS was quantified, as described in the legend to in Fig. 2*C*. Means ± S.D. ($n = 3$) of the fraction of cells with more JNK3 or ASK1 in the cytoplasm than in the nucleus also shown. (****, $p < 0.0001$). *D–F*, COS-7 cells were transfected with HA-ASK1, HA-MKK4, or GFP-JNK3 alone, or together with FLAG-tagged arrestin3 and its domains. Immunoprecipitation and Western blot analysis were performed as described in the legend to Fig. 2*A*.

nantly cytoplasmic in most cell types that express them endogenously, as well as in overexpressing HEK-293A cells (19, 20). The elimination of a putative NES or the addition of an engineered NES in arrestin1 or arrestin4 did not change their subcellular localization or their ability to redistribute JNK3 (19, 20). Although wild type (WT) arrestin2 did not move JNK3 from the nucleus, an engineered NES (point mutation Q394L) enabled it to redistribute JNK3 as efficiently as arrestin3 (18, 19). The elimination of NES in arrestin3 by the L393Q mutation only partially reduced its ability to recruit JNK3 to the cytoplasm (19). To test the effect of NES, we compared the ability of arrestin2-Q394L (NES+) and arrestin3-L393Q (NES−) to promote

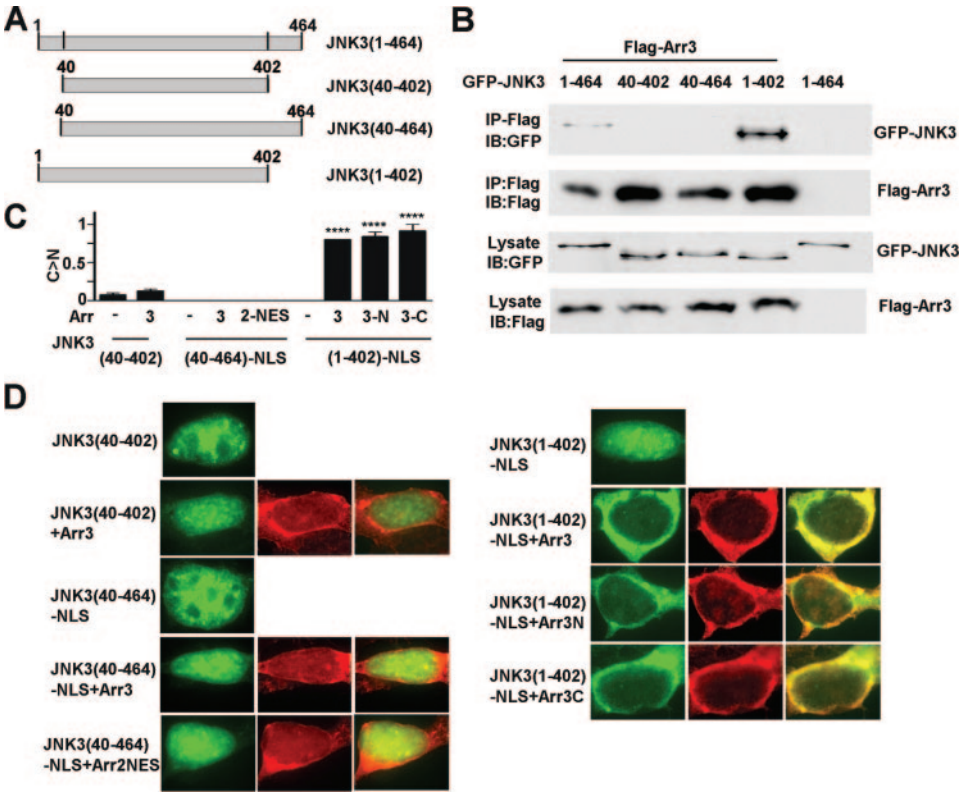

FIGURE 5. **N- and C termini of JNK3α2 regulate its binding to arrestin3.** *A*, Truncated JNK3 constructs have deletions in the N- (40–464) or C terminus (1–402), or in both termini (40–402). *B*, COS-7 cells were transfected GFP-tagged JNK3α2 constructs shown in *A* with FLAG-arrestin3 (FLAG-Arr3). Immunoprecipitation and Western blot analysis were performed as described in the legend to Fig. 2*A*. *C* and *D*, HEK-293A cells were transfected with GFP-JNK3-(40–402), GFP-JNK3-(40–464), or GFP-JNK3-(1–402) alone, or with FLAG-arrestin3 (Arr3), arrestin2 with engineered NES (Arr2NES), and N- (Arr3N) or C-domain (Arr3C) of arrestin3, respectively. Proteins were visualized, as described in the legend to Fig. 4. The localization of GFP-JNK3 constructs was quantified, as described in the legend to Fig. 2*C*. Means ± S.D. ($n = 3$) of the fraction of cells with more GFP-JNK3 in the cytoplasm than in the nucleus are shown. ****, $p < 0.0001$. Representative images are shown in *D*.

JNK3 activation with their corresponding parental proteins (Fig. 3*A*). The elimination of NES in arrestin3 reduced but did not abolish its ability to activate JNK3, whereas arrestin2-NES+ remained as ineffective as WT arrestin2. Thus, NES has only a minor effect on arrestin-dependent JNK3 activation (Fig. 3*A*).

Co-expression of arrestin3 with JNK3 and ASK1 increases phospho-JNK3 ~7-fold in COS-7 cells. To rule out a possible contribution of arrestin binding to endogenous receptors, we used arrestin mutant with a large deletion in the interdomain "hinge," which "freezes" the molecule in the basal conformation and severely impairs receptor binding (21, 25). Surprisingly, this mutant also yielded a strong (~9-fold) JNK3 activation, similar to WT arrestin3 (Fig. 3*B*). Thus, free arrestin3 can promote signal transduction from ASK1 to JNK3 in a receptor-independent fashion.

Structurally, arrestins are elongated molecules with N- and C-domains connected by a 12-residue hinge (23, 26–28). Arrestin domains are independent folding units that can be expressed separately and retain certain functions (19–21, 29–33). Separated domains of arrestin1 and arrestin4 bind JNK3 as efficiently as full-length proteins (19, 20). We found that it is also the case for both nonvisual arrestins (Fig. 4, *B* and *C*). Therefore, we tested the ability of individual domains to activate JNK3. Separately expressed N and C domains of arrestin3 only marginally increased phospho-JNK3 level (~2–3-fold) (Fig. 3*B*). Thus, the two domains in proper relative orientation are required for optimal JNK3 activation.

To investigate the possible interdependence of their binding to arrestin3, we expressed ASK1, MKK4, and JNK3 individually and in different combinations, immunoprecipitated FLAG-arrestin3, and immunoblotted for individual kinases (Fig. 2*A*). Among the three kinases, ASK1 demonstrated the highest level of binding and MKK4 the lowest. Similar amounts of ASK1 were found in complex with arrestin3 in all cases, indicating that downstream kinases do not appreciably affect arrestin3-ASK1 interaction. More JNK3 was detected in the complex upon co-expression with ASK1 than when JNK3 was expressed alone or with MKK4 (Fig. 2*A*), suggesting that ASK1 increases JNK3 binding to arrestin3. The amount of bound MKK4 increased upon co-expression with JNK3, ASK1, or both (Fig. 2*A*). Apparently, weak direct binding of MKK4 to arrestin3 is enhanced by its interactions with upstream and downstream kinases.

Separate N and C Domains of Arrestin3 Bind ASK1, MKK4, and JNK3—To identify arrestin elements involved in ASK1, MKK4, and JNK3 binding, we used both co-immunoprecipitation and nuclear exclusion assay. In HEK-293A cells, ASK1-NLS is exclusively nuclear (Fig. 4*A*). WT JNK3α2 spontaneously localizes to the nucleus. Individual domains of arrestin3 co-expressed with ASK1-NLS or JNK3 move the kinases to the cytoplasm as efficiently as the full-length arrestin3 (Fig. 4, *A* and *B*). Arrestin3 and its domains also co-immunoprecipitate ASK1, MKK4, and JNK3 (Fig. 4, *D–F*). Thus, each interacting kinase invariably engages both arrestin domains.

The N Terminus of JNK3 Interacts with Both Domains of Arrestin3—To map arrestin-binding elements of JNK3α2, we used three truncated forms with deletion of the N (JNK3-(40–464)) or C terminus (JNK3-(1–402)) or both (JNK3-(40–402)) (Fig. 5*A*). Co-immunoprecipitation revealed interaction of arrestin3 with full-length JNK3. The deletion of the N terminus or both termini almost completely eliminates the binding to arrestin3, whereas the deletion of the C terminus appears to enhance the binding, compared with full length JNK3 (Fig. 5*B*). To confirm these results by an independent method, we used a nuclear exclusion assay. JNK3-(40–402) spontaneously localized to the nucleus, and arrestin3 did not move it to the cytoplasm. JNK3-(40–464) was cytoplasmic, but with engineered NLS it became nuclear. Neither arrestin3 nor arrestin2-NES+

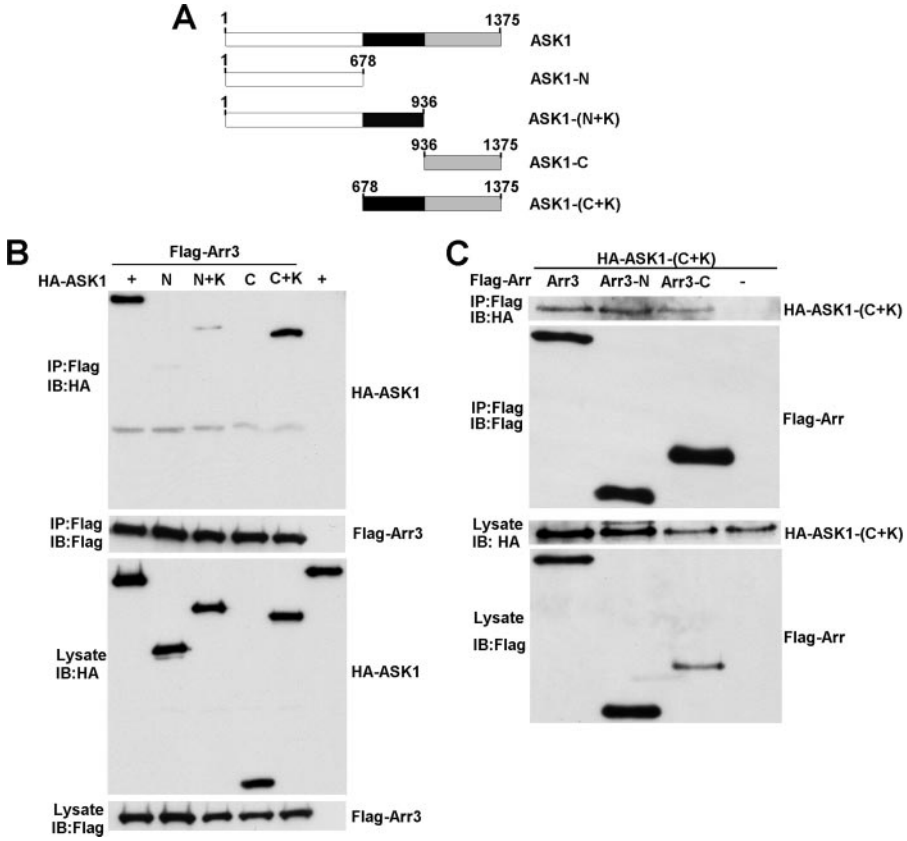

FIGURE 6. **Kinase and C-domains are the key arrestin-binding elements in ASK1.** *A*, Constructs containing different domains of ASK1: N domain (ASK1-N), N and Kinase domains (ASK1-(N+K)), C domain (ASK1-C), C and kinase domains (ASK1-(C+K)). *B* and *C*, COS-7 cells were transfected with HA-tagged ASK1 constructs shown in *panel A*, together with FLAG-arrestin3 (FLAG-Arr3) (*panel B*), or HA-ASK1-(C+K) with full-length arrestin3 (Arr3) or its N- (Arr3-N) and C-domain (Arr3-C) (*panel C*), respectively. Immunoprecipitation and Western blot analysis were performed as described in the legend to Fig. 2*A*.

(which expresses at much higher level and robustly binds full-length JNK3) moved it to the cytoplasm, suggesting that the N terminus is crucial for JNK3 binding to both arrestins. In contrast, arrestin3 and its domains effectively moved JNK3-(1–402)-NLS to the cytoplasm (Fig. 5, *C* and *D*). Thus, two different methods showed that the N terminus of JNK3α2 plays critical role in the interaction.

The C and Kinase Domains of ASK1 Interact with Arrestin3—To identify the elements of ASK1 mediating its binding to arrestin3, we expressed previously characterized (34) separate N, kinase (K), and C domains (Fig. 6*A*). We found that ASK1-N, ASK1-(N+K), ASK1-C all demonstrated much weaker binding to arrestin3 than full-length ASK1. In contrast, the binding of ASK1-(C+K) was comparable with that of full-length ASK1, indicating that the C and K domains contain key arrestin-binding elements (Fig. 6*B*). This part of ASK1 also interacts with individual domains of arrestin3 (Fig. 6*C*). ASK1 in the resting state forms homodimers through its C-terminal coiled-coil domain (35), although the kinase domain also participates in dimerization (36). Because both elements of ASK1 mediating its dimerization are necessary for optimal arrestin binding, it is likely that the ASK1 dimer engages the two domains of arrestin3.

Collectively these data indicate that each of the three kinases in the ASK1-MKK4-JNK3 cascade interacts with both arrestin domains. This implies the arrangement of the complex that is dramatically different from the one envisioned previously (17). To determine whether this arrangement of the kinases on the arrestin scaffold is a unique feature of the JNK3-activating module, we tested arrestin elements mediating its interactions with members of the c-Raf-1-MEK1-ERK2 cascade.

The Molecular Interactions Mediating Arrestin-dependent Assembly of the ERK Signaling Module—To determine the orientation of c-Raf-1, MEK1, and ERK2 on nonvisual arrestins, we co-expressed these kinases with arrestin2, arrestin3, and their separated N and C domains in COS-7 cells. We tested the ability of arrestins to co-immunoprecipitate the kinases, as well as the ability of c-Raf-1, MEK1, and ERK2 to co-immunoprecipitate arrestins and their separately expressed domains (Fig. 7). MEK1 (Fig. 7*B*) and c-Raf-1 (Fig. 7*C*) were readily detectable in complex with full-length arrestins. Both kinases demonstrate comparable binding to arrestin2, arrestin3, and individual domains. In contrast, the affinity of the ERK2-arrestin interaction appears to be lower than that of the upstream kinases, so that cross-linking was required for reliable detection of arrestin-ERK2 interaction (Fig. 7*A*). ERK2 also interacts comparably with both nonvisual arrestins and their N and C domains (Fig. 7*A*). Collectively, the data demonstrate that each of the three kinases, c-Raf-1, MEK1, and ERK2, binds equally well to both domains of arrestins. Thus, the members of this MAPK cascade assemble on the arrestin molecule similarly to the components of the ASK1-MKK4-JNK3 signaling module (Fig. 4). Therefore, this appears to be a universal mode of assembly of the three kinases on arrestin scaffolds.

DISCUSSION

Mitogen-activated protein kinases respond to a wide range of stimuli and regulate vital cellular functions: gene expression, proliferation, motility, metabolism, and apoptosis (37, 38). Arrestins were shown to scaffold three MAPK signaling modules that activate ERK1/2 (8), JNK3 (9), and p38 (15, 16). MAPK cascades consist of three protein kinases, MAPKKK, MAPKK, and MAPK, where each kinase is activated via phosphorylation by the one immediately upstream. Mammals have 11 MAPK, 7 MAPKK, and 21 MAPKKK genes (37, 38). Two different mechanisms control the efficiency and fidelity of MAPK signaling: recognition motifs used by kinases in binary interactions and scaffolds that organize them into multiprotein complexes. Scaf-

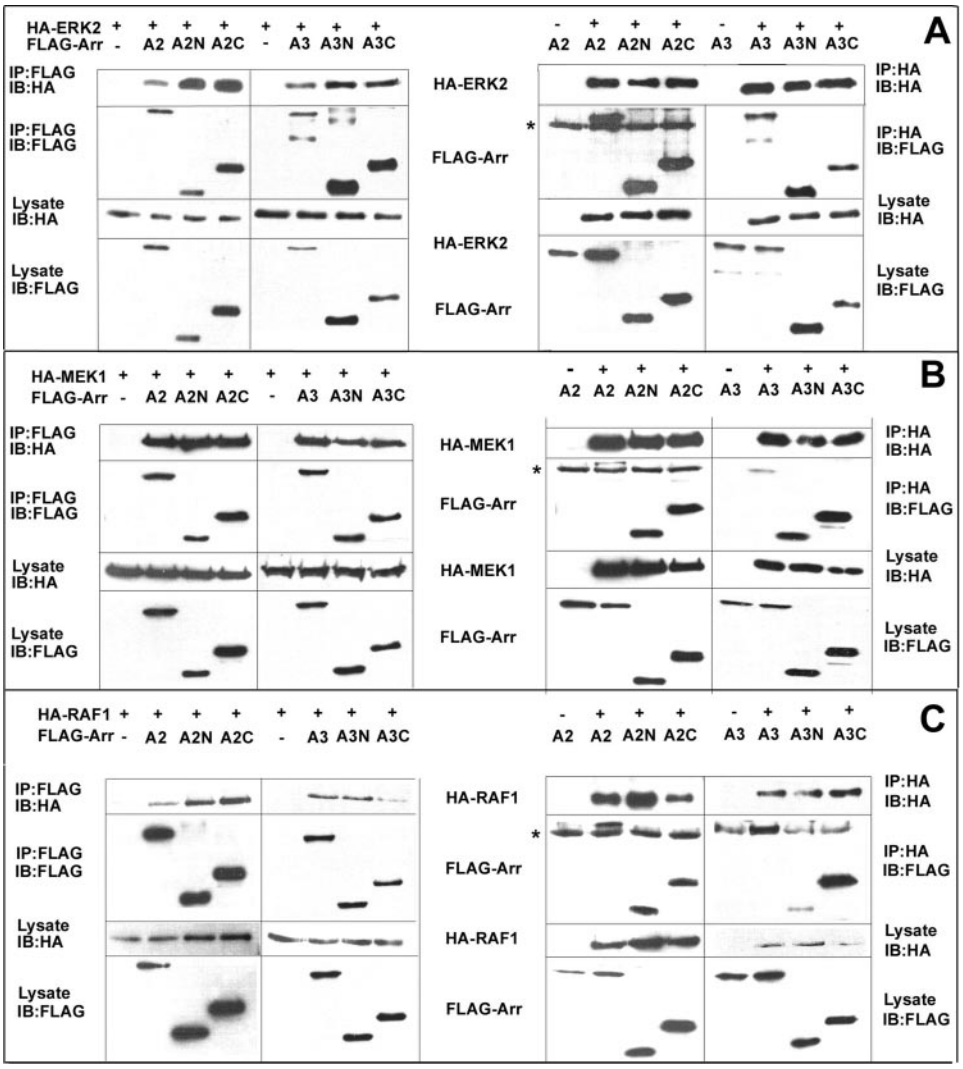

FIGURE 7. **ERK-2, MEK-1 and c-Raf-1 interact with both domains of arrestin2 and arrestin3.** COS-7 cells were transfected with plasmids encoding FLAG-tagged arrestin2 (A2), arrestin2 N-domain (A2N), arrestin2 C-domain (A2C), arrestin3 (A3), arrestin3 N-domain (A3N), or arrestin3 C-domain (A3C). HA-tagged ERK2, MEK1, and c-Raf1 were co-expressed with indicated FLAG-tagged arrestins. Arrestins were immunoprecipitated with M2 anti-FLAG antibody, and precipitates were probed for ERK2-HA (*A*), MEK1-HA (*B*) or c-Raf-1-HA (*C*). Alternatively, individually expressed kinases were immunoprecipitated with anti-HA antibody, and the precipitates were probed for arrestins using anti-FLAG antibody (*A–C*). The relative expression of each protein was confirmed by immunoblotting cell lysates (shown in the two lower blots in each panel). *Asterisks*, nonspecific band (heavy chain of the antibody).

folding proteins promote signaling between proper partners and likely prevent improper cross-talk and/or the assembly of nonfunctional complexes of mismatched kinases. Multiple scaffolding proteins, such as kinase suppressor of Ras (KSR) for ERK1/2 and JNK-interacting protein (JIP) for JNK3, have been identified (39). Arrestins were reported to serve as unconventional GPCR activation-dependent scaffolds (2, 40), but the molecular organization of the arrestin-MAPKKK-MAPKK-MAPK complex was not elucidated. Therefore, we investigated the assembly of the ASK1-MKK4-JNK3 and c-Raf-1-MEK1-ERK1/2 signaling complexes on arrestin proteins.

The Binding and Activation of MAP Kinases Are Distinct Functions of Arrestin Proteins—Previous studies have proposed that arrestin3, but not arrestin2, enhances JNK3 activation, because only this isoform interacts with ASK1 and JNK3, suggesting that ASK1 and JNK3 bind the N and C domain of arrestin3, respectively, whereas MKK4 is recruited via interactions with ASK1 and JNK3 (9, 17). However, the arrestin3-specific sequence that was proposed to mediate exclusive JNK3 interaction (17) was found to be unique for the rat protein (6). Moreover, subsequent studies showed that all arrestins bind JNK3 as avidly as arrestin3 (19, 20), ruling out this explanation for the unique ability of arrestin3 to promote JNK3 activation. Conceivably, it could be explained by the specific ability of arrestin3 to bind upstream kinases or by its preferential localization in subcellular compartments where the kinases of JNK3 activation cascade reside. Although all mammalian arrestins preferentially localize to the cytoplasm in most cells (19, 20, 41, 42), in neurons, arrestin3, in contrast to arrestin2, is enriched in synaptic membranes (43); it also carries a canonical NES in its C terminus (18). We found that WT and NES-arrestin3 enhance JNK3 activation, whereas WT and NES+ arrestin2 do not (Fig. 3*A*), indicating that NES is not crucial for JNK3 activation. Our finding that all vertebrate arrestins bind ASK1 (Fig. 2*C*) and MKK4 (Fig. 2*B*) shows that the ability to bind upstream kinases is not the feature of arrestin3 that determines its specificity.

Because all four arrestins bind ASK1, MKK4, and JNK3, apparently only arrestin3 holds them in a particular configuration conducive for signal transduction. Although the crystal structure of arrestin3 has not been solved, a remarkable structural conservation among the other three subtypes (23, 26–28, 44), along with considerable sequence homology in the arrestin family (1, 45, 46), suggests that the overall fold of the molecule must be similar. Therefore, subtle differences in the interactions with one or more of these kinases, mediated by arrestin3-specific residues exposed on the non-receptor-binding surface (Fig. 8), are likely responsible for the ability of arrestin3 to promote JNK3 activation. The molecular mechanisms of MAPK activation are usually complex. For example, unphosphorylated JNK3 assumes an open conformation in which some of the catalytic residues are misaligned (47). In addition, the phosphorylation lip partially blocks the substrate-binding site. Both global (domain closure to align catalytic residues) and local (lip refolding to allow substrate binding) conformational changes are required for JNK3 activation (47). Subtle structural differences between arrestins may not be

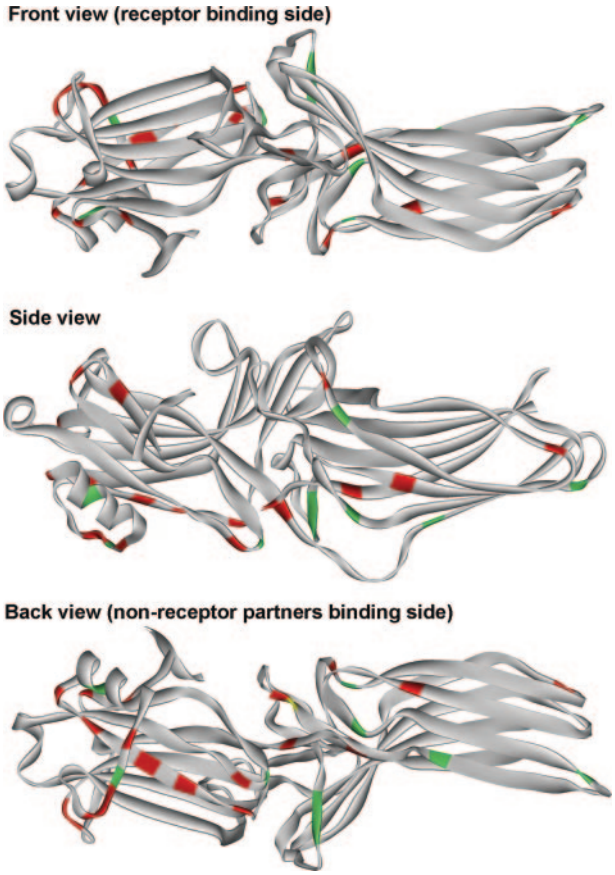

FIGURE 8. **Arrestin3-specific residues on the non-receptor-binding side of the molecule.** The residues that are specific for arrestin3 (absent in the other three mammalian subtypes) were identified based on sequence alignment in (1). The positions of residues that represent nonconservative and conservative substitutions (as compared with arrestin2) are highlighted in red and green, respectively. Because the structure of arrestin3 was not solved, high-resolution structure of arrestin2 (PDB ID 1G4M) (27) was used to generate this picture. Three views are shown: upper panel, receptor-binding side (view down the concave sides of both domains); middle panel, side view; and lower panel, non-receptor-binding "back" of the arrestin molecule where signaling molecules interacting with the arrestin-receptor complex bind.

fatal for the binding, but even a slight change in orientation of ASK1, MKK4, and/or JNK3 on arrestin might hinder the conformational changes in one or more kinases, making signal propagation impossible.

Is Receptor-dependent Enhancement of MAPK Activation Compatible with Receptor-independent Binding of MAP Kinases to Arrestin?—Arrestin-dependent JNK3 activation was first shown to be controlled by GRCR signaling (9), although it was also observed in the absence of receptor stimulation (17). The finding that receptor binding-impaired arrestin3 mutant with 7-residue deletion in the interdomain hinge (21, 25) effectively promotes JNK3 phosphorylation (Fig. 3*B*) indicates that free arrestin3 acts as a scaffold for the JNK3 activation cascade. Conceivably, the complexes of JNK3 and upstream kinases with free or receptor-bound arrestin3 coexist in cells, where cytoplasmic arrestin3-ASK1-MKK4-JNK3 complex generates certain level of JNK3 activity. How can this model be reconciled with reports that receptor stimulation significantly facilitates this process (9)? Mechanistically, arrestin recruitment to the receptor results in two important changes. First, receptor binding is accompanied by a global conformational change in arrestin (25, 30, 31, 48, 49) (reviewed in Ref. 3). This rearrangement likely changes the set of exposed arrestin elements, thereby altering the "attractiveness" of arrestin for different binding partners (50). This conformational change involves the movement of the two arrestin domains relative to each other (25). Because the binding sites for each kinase in the ASK1-MKK4-JNK3 and c-Raf-1-MEK1-ERK2 cascades span both arrestin domains, domain movement could significantly affect the relative orientation of the kinases in the complex, thereby changing the signaling efficiency. However, our finding that arrestin3 with a hinge deletion that severely impedes domain movement necessary for receptor binding (21, 25) effectively promotes JNK3 activation (Fig. 3*B*) makes this scenario unlikely. Second, massive recruitment of arrestin complexes with bound kinases to the receptor greatly increases their local concentration in the vicinity of the plasma membrane. Because MAPK signaling involves three different proteins, the law of mass action predicts enormous effects of concentration. This factor explains why overexpression of ASK1 yields essentially the same level of JNK3 activation as receptor stimulation in the presence of relatively low levels of endogenous MAPKKK (9, 17). However, arrestin2 binds all three components of the ASK1-MKK4-JNK3 cascade as efficiently as arrestin3 (Figs. 2, *B* and *C*, and 4*C*), and it also binds many of the same GPCRs (32, 42), yet it does not appreciably promote JNK3 activation (9) (Fig. 1). Thus, in addition to increased local concentration of the components, JNK3 activation requires a scaffold that arranges the three kinases in correct orientation. Apparently, arrestin2 cannot fulfill this latter function. Our findings that separated N and C domains of arrestin3 bind the components of the JNK3 cascade (Refs. 19 and 20; Fig. 4, *A*, *B*, and *D–F*), yet fail to increase the level of JNK3 phosphorylation (Fig. 3*B*), also support this idea.

Based on the existing data, we propose the following two-step model of arrestin-dependent activation of MAP kinases. First, both of the free nonvisual arrestins bind the kinases in the cytoplasm, so that their recruitment to the active receptor dramatically increases the local concentration of all three components near receptor-rich membranes. Second, arrestin3, but not arrestin2, promotes accurate interactions between MAPKKK and MAPKK, as well as MAPKK and MAPK, in the JNK3 cascade. At this step arrestin2, which assembles the same kinases into an unproductive complex, serves as a "sink" suppressing JNK3 activation. In fact, mutual antagonism of the two nonvisual arrestins in MAPK activation has been reported (51). JNK3 is a neuron-specific isoform implicated in apoptotic cell death (52–55). Interestingly, during brain development, as cells progress from "disposable" precursors to "irreplaceable" postmitotic mature neurons, the expression of the two nonvisual arrestins changes from almost equal to an ~10–20-fold excess of arrestin2 over arrestin3 (56, 57). In view of our data, it is tempting to speculate that a dramatic increase in the arrestin2 level protects neurons from "accidental" death, making JNK3-initiated apoptosis less likely.

Biological Implications of the Relative Affinity of Different Kinases for Arrestin and the Size of the Signaling Complex—From a mechanistic viewpoint, the shape and stoichiometry of the complex, its stability, and the elements of all four proteins involved in their interactions are of utmost importance. Inter-

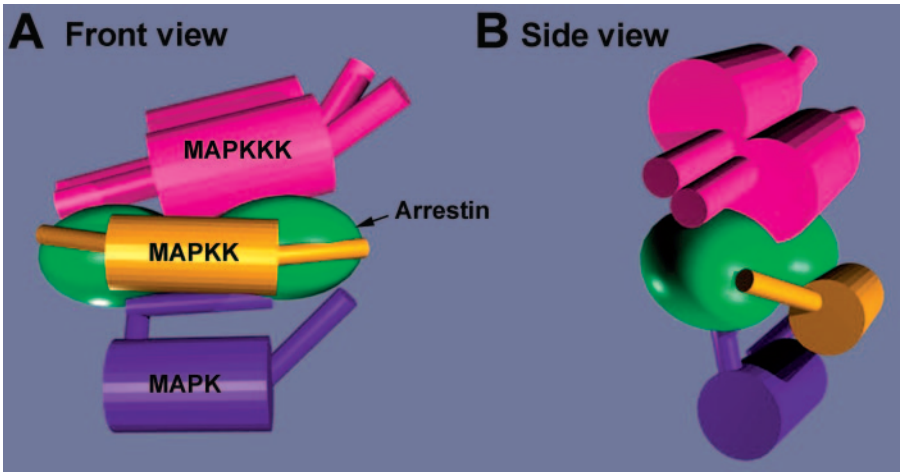

FIGURE 9. **Model of the MAPK signaling module organized by arrestin.** Front (*A*) and side (*B*) views of the three-dimensional model of arrestin complex with the three MAP kinases are shown. Arrestin (*green*) is shown as an elongated two-domain molecule. All the kinases are shown with thick cylinders representing kinase domain, and thin ones representing N- and C-terminal elements. MAPKKK (*pink*) is represented as ASK1 dimer in the JNK3 pathway with its C and kinase domain interacting with arrestin, and MAPK (*purple*) with its N terminus interacting with arrestin. MAPKK (*yellow*) also interacts with both domains of arrestin.

estingly, the relative affinities of these interactions in the two cascades are different. MKK4, MAPKK in the JNK3-activating module, binds arrestin with lower affinity than ASK1 and JNK3. The presence of JNK3, ASK1, or both enhances MKK4 binding, revealing the role of other kinases in its recruitment. In addition, the presence of ASK1 enhances JNK3 binding to arrestin3. A comparison of the relative sizes of arrestins and their interaction partners suggests that few proteins can be simultaneously accommodated by a single arrestin or arrestin-receptor complex (6). Considering that arrestins assemble three different cascades activating JNK3, ERK1/2, and p38, independent binding of MAPKKK, MAPKK, and MAPK would make the probability of simultaneous association of mismatched kinases very high. Therefore, the observed interdependence of the binding of kinases of the same cascade likely serves to ensure the assembly of productive complexes containing matching kinases.

In contrast to the JNK3 module, in the ERK cascade c-Raf-1 and MEK1 demonstrate robust binding, whereas arrestin interaction with ERK2 is much weaker. These subtle differences suggest that, activated in an arrestin-dependent manner, JNK3 is more likely to remain in the complex and to phosphorylate cytoplasmic substrates (as proposed by McDonald *et al.* (9)) than ERK2, although this pattern may well be different with free (this study) and receptor-bound (8, 9, 58) arrestins. The outcome of arrestin-dependent MAPK activation could also depend on the particular GPCR involved (59, 60) and/or on the state of receptor oligomerization (61). Recent studies show that only monomeric arrestin binds receptors and that each individual receptor molecule binds arrestin (62, 63). Thus, the GPCR oligomer, as well as large arrays of monomeric receptors forming in coated pits during internalization, would bring multiple molecules of bound arrestin in close proximity, potentially creating large scaffolds with distinct signaling capabilities (64, 65).

Free arrestins also oligomerize, spontaneously (62, 66, 67) or with the help of physiological concentrations of inositol hexakisphosphate (68, 69), raising the question of whether MAPK cascades are organized by a single arrestin molecule or an oligomer. Considering that arrestins mobilize MAP kinases to GPCRs, only the non-receptor-binding side of the molecule remains available for interactions with kinases (70). In all of the proposed structures of arrestin oligomers these surfaces face away from each other (62, 68, 71), suggesting that even if oligomers play a role, the "back" of a single arrestin molecule (Fig. 8) likely holds all three kinases.

Model of MAPKKK-MAPKK-MAPK Signaling Module Assembled on the Arrestin Scaffold—Proposed models of the assembly of JNK3 (9) and ERK1/2 (8) activation cascades on arrestins are incompatible with our findings that each interacting kinase invariably engages both arrestin domains (Figs. 4 and 7). Arrestin3 preferentially binds the longest isoform, JNK3α2 (9). Because the JNK3 isoforms share essentially the same kinase domain and differ in their N and C termini, it was hardly surprising to find that the termini of JNK3α2 regulate arrestin binding (Fig. 5). We found that an intact JNK3α2 N terminus is crucial for arrestin3 interaction, in agreement with a previous report (72). Because C-terminally truncated JNK3 binds the separated N and C domains of arrestin3 (Fig. 5, *C* and *D*), the binding site for the N terminus of JNK3 spans both domains. We also found that N-terminally truncated ASK1, containing only the C and kinase domains, binds arrestin3 essentially as efficiently as full-length ASK1, similarly engaging both arrestin domains (Fig. 6).

Based on our identification of multiple arrestin-binding elements in MAP kinases and localization of kinase-binding elements in arrestins, we propose a model of arrestin-dependent assembly of the MAPK signaling module. Arrestin binds all three kinases, assembling MAPKKK, MAPKK, and MAPK along its short axis, with each kinase directly interacting with both domains of arrestin. Fig. 9 shows dimeric MAPKKK, which represents ASK1 in the JNK3 activation module. Monomeric c-Raf-1 likely occupies this position in the ERK activation cascade. The relative size of arrestin and the kinases suggests that an individual arrestin molecule can assemble only one MAPK signaling module. Enhanced binding of JNK3 and MKK4 in the presence of ASK1 indicates that additional interactions between kinases of a particular cascade make their binding cooperative, thereby precluding the formation of unproductive complexes with mismatched kinases.

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