β-Arrestins Regulate Mitogenic Signaling and Clathrin-mediated Endocytosis of the Insulin-like Growth Factor I Receptor*

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β-Arrestins mediate agonist-dependent desensitization of G protein-coupled receptors and target the receptors to clathrin-coated pits for internalization. Here we report an expanded role of β-arrestins in promoting clathrin-mediated endocytosis of a tyrosine kinase growth factor receptor, i.e. the insulin-like growth factor I (IGF-1) receptor. β-Arrestins bind to the ligand-occupied IGF-1 receptors, promote their endocytosis, and enhance IGF-1-dependent mitogen-activated protein kinase phosphorylation and DNA synthesis. Our results suggest a role for β-arrestins in regulating mitogenic signaling and clathrin-mediated endocytosis of receptors not classically coupled to G proteins.

Many cell surface receptors undergo endocytosis via clathrin-coated vesicles (1). Whereas some receptors, such as the transferrin receptor and low density lipoprotein receptor, are constitutively recycling, other receptors, such as many tyrosine kinase growth factor receptors and G protein-coupled receptors (GPCRs), are internalized only after binding to their respective agonists (2, 3). In the category of GPCRs, recent evidence with the β2-adrenergic receptor (4) and M2 muscarinic cholinergic receptor (5) has suggested that β-arrestins, including β-arrestin1 (β-arrestin) and β-arrestin2 (arrestin3), function as clathrin adaptors, which target the ligand-occupied receptors into clathrin-coated pits for internalization. Following phosphorylation of the agonist-occupied receptors by G protein-coupled receptor kinases β-arrestins bind to the receptors, thereby uncoupling the receptors from G proteins and causing rapid desensitization (6–8). Subsequently, β-arrestins promote receptor endocytosis into endosomal vesicles, wherein the receptors are either dephosphorylated and recycled back to the plasma membrane or targeted for degradation (9, 10).

Recent evidence has suggested that agonist-promoted endocytosis plays a dual regulatory role in receptor-mediated signaling pathways of both tyrosine kinase growth factor receptors and GPCRs. On the one hand, it attenuates some forms of extracellular signaling by reducing the number of cell surface receptors; on the other hand, it is critical for transducing some agonist-dependent intracellular responses. For example, it has been shown that receptor endocytosis is required for agonist-induced mitogenic signaling of various tyrosine kinase growth factor receptors, such as the receptors for epidermal growth factor receptor (11), nerve growth factor (12), and IGF-1 (13), as well as GPCRs, such as the β2-adrenergic receptor and receptors for lysophosphatic acid, thrombin, and bombesin (14, 15). Thus, inhibition of internalization of these receptors reduces agonist-dependent MAP kinase activation.

The IGF-1 receptor is composed of a heterotetrameric αβ2 structure and possesses ligand-induced tyrosine kinase activity (16). The activated receptor catalyzes both receptor autophosphorylation and tyrosine phosphorylation of exogenous proteins, including insulin receptor substrate-1 (IRS-1) (17, 18) and -2 (IRS-2) (19) and the Shc proteins (20). Previous studies have suggested that a number of the biochemical signaling properties of the IGF-1 receptor are mediated through classical GPCR signaling pathways. For example, activation of MAP kinase by IGF-1 appears to utilize a Gβγ-mediated pathway essentially identical to that utilized by Gαi-coupled lysophosphatic acid receptors (21). Thus, IGF-1 stimulation of MAP kinase phosphorylation in Rat-1 cells is blocked by pertussis toxin or by a Gβγ sequestering reagent, the carboxyl terminus of G protein-coupled receptor kinase 2.

Based on the extensive structural homology to the insulin receptor, the ligand-occupied IGF-1 receptor is believed to utilize clathrin-coated vesicles for internalization (22, 23). Because β-arrestins act as clathrin adaptors in GPCR endocytosis, we considered the possibility that β-arrestins might play a broader role in clathrin-mediated internalization of receptors other than those classically coupled to G proteins (heptahelical receptors).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The stable cell lines were generated as described (24). Only low passaged cells (less than 15 passages) were used in this study.

Immunoprecipitation and Western Blot Analysis—HEK 293 cells overexpressing the IGF-1 receptor and either form of β-arrestin were treated or not with 10 nM IGF-1 (Sigma), cross-linked or not with dithiobis(succinimidyl propionate) (DSP, Pierce) as described (25) and harvested in lysis buffer (24) containing 1 mM sodium vanadate, 10 mM dithiobis(succinimidyl propionate) (DSP, Pierce) as described (25) and harvested in lysis buffer (24) containing 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and protease inhibitor mixtures. The IGF-1 receptor was immunoprecipitated using a monoclonal antibody specific to its α subunit (Calbiochem), and the proteins were resolved by SDS-PAGE. Western blot analysis was performed using antibodies against both β-arrestin1 and β-arrestin2. The phosphorylated IGF-1 receptor was detected by an anti-phosphotyrosine antibody (RC20H, Transduction Labs). These immunoblots were visualized by ECL (Amersham Pharmacia Biotech). The nitrocellulose membranes were stripped and reprobed using a polyclonal antibody specific to the β subunit of the IGF-1 receptor (Santa Cruz Biotech) to confirm equal expression of the IGF-1 receptor in each sample. The expression of K44A dynamin was detected by probing its immunoblot with a dynamin antibody (Transduction Labs). To detect tyrosine phosphorylation of Gab1, HEK 293 cells were co-transfected with pCDNA3/Gab1 and either a control vector or a β-arrestin expression plasmid. Cells were treated with IGF-1 or not for 5 min and were lysed in RIPA buffer (15) with 1 mM sodium vanadate and protease inhibitors. Comparably expressed Gab1 was immunoprecipitated with an antibody specific to the Gab1 carboxyl terminus.
with b or b eitheringly increased co-immunoprecipitation of the receptor with receptor in HEK 293 cells. As shown in Fig. 1, IGF-1 treatment for 0, 1, and 3 min. The agonist-promoted association of the IGF-1 receptor with amino acids 23–189 of Gab1 and resolved by SDS-PAGE. The immunoblot was developed with an anti-phosphotyrosine antibody as described. The endogenous β-arrestin1 (a) or β-arrestin2 (b) as shown in the upper panel. The lower panel is an immunoblot of β-arrestin1 (a) or β-arrestin2 (b) representing 5% of whole cell lysates. IP, immunoprecipitation. c, HEK 293 cells stably transfected with the IGF-1 receptor were serum-starved for 90 min followed by IGF-1 treatment for 0, 1, and 3 min. The upper panel shows the endogenous β-arrestin1 and β-arrestin2 co-immunoprecipitated with the IGF-1 receptor. The lower panel is the immunoblot of both endogenous β-arrestin1 and β-arrestin2 from 5% of the whole cell lysates.

raised against a glutathione S-transferase fusion protein comprising amino acids 23–189 of Gab1 and resolved by SDS-PAGE. The immunoblot was developed with an anti-phosphotyrosine antibody as described. IGF-1 Receptor Internalization Determined by Flow Cytometry—Agonist-induced IGF-1 receptor internalization was determined by immunofluorescence flow cytometry analysis similar to that described for the β2-adrenergic receptor (26). 2 days after transfection, cells were starved for 90 min and then treated with or without 10 nM IGF-1 at 37 °C for 20 min. Next, cells were incubated on ice for 1 h with a monoclonal antibody specific to the α subunit of the IGF-1 receptor, resolved by SDS-PAGE, and immunoblot was then stripped and reprobed with both anti-phosphotyrosine antibody as shown in the top panel. This immunoblot was then stripped and reprobed with both anti-βarr1 and anti-βarr2 antibodies as shown in the middle panel. The bottom panel is an immunoblot of different β-arrestins representing 5% of the cell lysates. b, HEK 293 cells were transiently transfected with the IGF-1 receptor and either a control vector or a β-arrestin expression plasmid. Cells were starved for 90 min and then treated with or without IGF-1 at 37 °C for 20 min. The plasma membrane content of IGF-1 receptors was determined by flow cytometry analysis. The agonist-promoted IGF-1 receptor internalization was expressed as the percentage of IGF-1-induced cell surface receptor loss. Values represent the means ± S.E. from five independent experiments done by duplicated samples.

RESULTS AND DISCUSSION

To investigate the potential functional role of β-arrestins in clathrin-mediated endocytosis of the IGF-1 receptor, we first examined the ability of β-arrestins to interact with the IGF-1 receptor in HEK 293 cells. As shown in Fig. 1, IGF-1 treatment for 2 min followed by covalent cross-linking with DSP strikingly increased co-immunoprecipitation of the receptor with either β-arrestin1 (Fig. 1a) or β-arrestin2 (Fig. 1b) in cells overexpressing IGF-1 receptors and either form of β-arrestin. Agonist-promoted association of the IGF-1 receptor with endogenous β-arrestin1 and β-arrestin2 could also be detected in cells overexpressing IGF-1 receptors alone (Fig. 1c).

Previously we have demonstrated that phosphorylation/dephosphorylation of β-arrestin1 regulates clathrin-mediated endocytosis of the β2-adrenergic receptor (24). Some of the biological functions of β-arrestin1 appear to be controlled by phosphorylation at a single serine, Ser-412, located close to the carboxyl terminus. Cytosolic β-arrestin1 is phosphorylated, and although competent to bind receptors, it is deficient in mediating downstream functions such as clathrin binding. Dephosphorylation of the β-arrestin1 occurs at the plasma membrane and is required for its activity to promote receptor internalization. The S412A and S412D mutants, which, respectively, mimic the dephosphorylated and phosphorylated forms of β-arrestin1, have been proven to be useful probes for exploring the roles of β-arrestin1 in GPCR function. The S412A mutant is generally more active in promoting GPCR internalization, whereas the S412D mutant serves as a dominant-negative.

As shown in Fig. 2a, transfection of either wild-type or mutant β-arrestins in HEK 293 cells stably overexpressing the IGF-1 receptor did not affect agonist-dependent tyrosine phosphorylation of the receptors. However, IGF-1 treatment for 1 min resulted in increased association of the IGF-1 receptor with wild-type β-arrestins and Ser-412 mutants (S412A and S412D) of β-arrestin1.

Next, we assessed the effect of β-arrestins on IGF-1 receptor internalization in HEK 293 cells. Incubation with IGF-1 for 20 min caused ~25% loss of cell surface IGF-1 receptors in control cells (Fig. 2b). Overexpression of either wild-type β-arrestin1 or β-arrestin2 increased the agonist-dependent loss of cell surface...
receptors. S412A β-arrestin1 enhanced IGF-1 receptor internalization even further. In contrast, S412D β-arrestin1, which functions as a dominant-negative inhibitor of β-arrestin-mediated GPCR endocytosis, significantly reduced endocytosis of the IGF-1 receptor. Similar to other receptors that are internalized through clathrin-coated vesicles, the IGF-1 receptor internalization was impaired by overexpression of the K44A mutant of dynamin (Fig. 3a).

Previously it has been shown that the process of receptor internalization plays an essential role in receptor-mediated mitogenic signaling (11–15). Similarly, inhibition of IGF-1 receptor internalization by the K44A mutant of dynamin significantly impaired IGF-1-stimulated MAP kinase phosphorylation (Fig. 3b). Considering the role of β-arrestins in IGF-1 receptor internalization, we next investigated IGF-1 receptor-mediated mitogenic signaling. First, we examined MAP kinase phosphorylation in HEK 293 cells stably overexpressing β-arrestins. At endogenous receptor levels, IGF-1-stimulated MAP kinase phosphorylation was moderately increased by overexpression of wild-type β-arrestin1 or β-arrestin2 and dramatically enhanced by overexpression of S412A β-arrestin1 (Fig. 4, a and b). S412D β-arrestin1 acted as a dominant-negative mutant and significantly attenuated IGF-1-induced MAP kinase activation (Fig. 4, a and b). These results support the hypothesis that β-arrestin-mediated receptor endocytosis is required for MAP kinase activation via the IGF-1 receptor. Similar to the classical G coupled receptors, the IGF-1 receptor employs a mechanism involving both tyrosine phosphorylation and G subunits derived from pertussis toxin-sensitive G proteins in its MAP kinase signaling cascade (21). The enhancing effect of β-arrestins on IGF-1-stimulated MAP kinase activation was abolished by pertussis toxin (data not shown).

One of the earliest steps of the mitogenic signaling cascade triggered by the IGF-1 receptor is the receptor-catalyzed tyrosine phosphorylation of MAP kinase substrates in its MAP kinase signaling cascade (21).

**Fig. 3.** Effect of K44A dynamin on internalization of the IGF-1 receptor and IGF-1-stimulated MAP kinase phosphorylation. a, HEK 293 cells were transiently transfected with the IGF-1 receptor and either a control vector or a K44A dynamin expression plasmid. The assay of IGF-1 receptor internalization was performed. Values represent the means ± S.E. from two independent experiments done by duplicated samples. b, subconfluent cells as described above were starved overnight. Cells were treated with or without IGF-1 for 3 min and harvested. Levels of phosphorylated ERK1 and ERK2 were determined using an anti-active MAP kinase antibody as shown in the top panel. The immunoblot was then stripped and the total cellular ERK2 was detected by an anti-ERK2 antibody as shown in the middle panel. The bottom panel is an immunoblot of K44A dynamin. The figure shown is a representative result from three independent experiments.

**Fig. 4.** Effect of β-arrestins on IGF-1 receptor-mediated MAP kinase phosphorylation. a, subconfluent HEK 293 cells stably overexpressing a control vector (mock), β-arrestin2, or β-arrestin1 (wild type, S412A, and S412D) were starved overnight. Cells were treated with or without IGF-1 for 3 min and harvested. Levels of phosphorylated ERK1 and ERK2 and total cellular ERK2 was determined as described above. The bottom panel is the same immunoblot stripped and reprobed with both anti-βarr1 and anti-βarr2 antibodies. b, IGF-1-promoted MAP kinase phosphorylation was determined. Data shown represent the means ± S.E. from four independent experiments. c, HEK 293 cells were co-transfected with pCDNA3 Gab1 plasmid and either an empty vector or a β-arrestin expression plasmid. Cells were starved overnight and incubated with or without IGF-1 for 5 min before lysis in RIPA buffer. Equally expressed Gab1 protein was immunoprecipitated, resolved by SDS-PAGE, and detected by an anti-phosphotyrosine antibody. Data shown are the means ± S.E. from three independent experiments. The bottom panel is an immunoblot of Gab1 tyrosine phosphorylation.

**Fig. 5.** Effect of β-arrestins on IGF-1 receptor-mediated DNA synthesis. HEK 293 cells stably overexpressing wild-type or mutant β-arrestins were serum-starved for 30 h followed by the addition or not of IGF-1 for 16 h. [3H]Thymidine (1 μCi/ml) was added for 1 h, and then cells were harvested as described. Data shown represent the means ± S.E. from three independent experiments done by duplicated samples.
sine phosphorylation of IRS-1 and Shc proteins (17–20). Recently it has been shown that the activated insulin receptor phosphorylates the Grb2-associated binder 1 (Gab1), which binds to and activates phosphatidylinositol 3-kinase (27). Phosphatidylinositol 3-kinase activity has been implicated in the G1-mediated MAP kinase signaling cascade upstream of Ras (28–30). To determine the step in the MAP kinase signaling pathway at which β-arrestin-mediated receptor internalization is required, we examined IGF-1-stimulated tyrosine phosphorylation of IRS-1, Shc, and Gab1 proteins in HEK 293 cells overexpressing β-arrestins. We observed that the extent of IGF-1-induced tyrosine phosphorylation of IRS-1 and Shc proteins was not significantly altered by overexpression of wild-type or mutant β-arrestins (data not shown). Similar results have been reported for the tyrosine phosphorylation of Shc proteins mediated by the β2-adrenergic receptor (15). In contrast, as shown in Fig. 4c, IGF-1-induced Gab1 tyrosine phosphorylation was enhanced by overexpressing S412A β-arrestin1 but was impaired by overexpressing S412D β-arrestin1. This result suggests that β-arrestin-dependent internalization of the IGF-1 receptor is necessary for tyrosine phosphorylation of Gab1 and that Gab1 may serve as one of the important signaling intermediates in the IGF-1-mediated MAP kinase activation pathway.

In agreement with our results, Chow et al. recently reported that inhibition of IGF-1 receptor internalization in CHO cells by a chemical reagent, i.e. dansylcadaverine, impaired MAP kinase activation but not tyrosine phosphorylation of the receptor and IRS-1 (13). In contrast, however, they found that IGF-1-induced Shc tyrosine phosphorylation was reduced in the presence of dansylcadaverine. The reason for this apparent discrepancy is not yet clear, although it might relate to distinct mechanisms by which different reagents inhibit receptor endocytosis. The S412D β-arrestin1 mutant inhibits receptor endocytosis by interfering with receptor targeting to clathrin-coated pits (26). The mechanism of dansylcadaverine is not fully understood, although some evidence indicates that it inhibits receptor trafficking at a step proximal to the formation of endocytotic vesicles (31, 32). It is possible that agonist-dependent tyrosine phosphorylation of Shc proteins proceeds at an earlier step that is inhibited by dansylcadaverine, but this is prior to β-arrestin-mediated receptor targeting to clathrin-coated pits, whereas Gab1 phosphorylation following IGF-1 stimulation occurs thereafter.

To further investigate the regulatory role of β-arrestins in IGF-1 receptor-mediated mitogenic signaling, we assessed their effects on IGF-1-stimulated DNA synthesis in these HEK 293 cell lines overexpressing β-arrestins. As shown in Fig. 5, IGF-1-dependent [3H]thymidine incorporation was significantly increased by overexpression of S412A β-arrestin1, followed by β-arrestin2 and β-arrestin1 but was inhibited by overexpression of S412D β-arrestin1. Consistent with the effects of β-arrestins on the activation of MAP kinases, this result suggests that IGF-1 receptor endocytosis is also required for IGF-1-stimulated DNA synthesis.

Taken together, our findings suggest that β-arrestins may play a much wider role in receptor biology than had been previously imagined. In addition to their effects on internalization and signaling of GPCRs, they may be involved in regulating the function of a variety of receptor tyrosine kinases and other surface membrane receptors in response to ligand stimulation.

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