 Src-mediated Tyrosine Phosphorylation of Dynamin Is Required for β2-Adrenergic Receptor Internalization and Mitogen-activated Protein Kinase Signaling*

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Some forms of G protein-coupled receptor signaling, such as activation of mitogen-activated protein kinase cascade as well as resensitization of receptors after hormone-induced desensitization, require receptor internalization via dynamin-dependent clathrin-coated pit mechanisms. Here we demonstrate that activation of β2-adrenergic receptors (β2-ARs) leads to c-Src-mediated tyrosine phosphorylation of dynamin, which is required for receptor internalization. Two tyrosine residues, Tyr231 and Tyr597, are identified as the major phosphorylation sites. Mutation of these residues to phenylalanine dramatically decreases the c-Src-mediated phosphorylation of dynamin following β2-AR stimulation. Moreover, expression of Y231F/Y597F dynamin inhibits β2-AR internalization and the isoproterenol-stimulated mitogen-activated protein kinase activation. Thus, agonist-induced, c-Src-mediated tyrosine phosphorylation of dynamin is essential for its function in clathrin-mediated G protein-coupled receptor endocytosis.

Agonist stimulation of cell surface receptors results in rapid attenuation of receptor responsiveness, a process termed desensitization. In the case of G protein-coupled receptors (GPCRs),1 this process is initiated by phosphorylation of agonist-occupied receptors by the G protein-coupled receptor kinase family (1) and the subsequent formation of high affinity β-arrestin bound, desensitized GPCRs then undergo β-arrestin-mediated targeting to clathrin-coated pits (3, 4), wherein they are sequestered, internalized, and ultimately either recycled to the cell surface or targeted for degradation. Interestingly, the process of GPCR internalization also plays a critical, albeit poorly understood, role in some aspects of GPCR signal transduction, e.g. MAP kinase activation (5, 6).

The process of cell surface receptor internalization (also termed endocytosis) is dependent on the invagination and fusion of clathrin-coated vesicles from the plasma membrane into the cytosol. Endocytosis of many GPCRs and receptor tyrosine kinases (RTKs) requires the GTPase activity of dynamin. Receptor stimulation leads to recruitment of cytosolic dynamin to coated pits where it induces constriction of the pits and fission of vesicles (7, 8). In vitro, dynamin is activated by a variety of mechanisms including binding to microtubules (9), phospholipids (10), and several proteins containing SH3 domains (11). Collectively, these molecules are thought to induce a conformational change in dynamin, inducing homo-oligomerization and subsequent increase in GTPase activity (12). Recently, dynamin was shown to contain a GTPase effector domain that interacts with its N-terminal GTPase domain to stimulate GTP hydrolysis (13). GTPase defective dynamin mutants (e.g. K44A) specifically block endocytic coated vesicle formation and agonist-mediated internalization of GPCRs and RTKs (14, 15).

Accumulating evidence suggests that tyrosine protein phosphorylation is critical for the internalization of cell surface receptors. Exposure of cells to tyrosine kinase inhibitors profoundly attenuates cross-linking-induced internalization of B cell receptors (16) and hormone-induced internalization of tyrosine kinase growth factor receptors (17). Recently, overexpression of the nonreceptor tyrosine kinase c-Src was found to cause an increase in the internalization rate constant of the epidermal growth factor (EGF) receptor following EGF treatment (18). Although these results suggest involvement of tyrosine kinases in the process of receptor internalization, their role(s) in this process is not well defined. Therefore, we tested the hypothesis that tyrosine kinase activity might regulate endocytosis by acting on accessory molecules important for receptor internalization such as dynamin. We find not only that Src-mediated tyrosine phosphorylation of dynamin is required for β2-AR internalization but that this phosphorylation event itself is regulated by receptor stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293 cells were maintained and transfected precisely as described (5, 6). All assays were performed 40 h after transfection. HEK293 cells stably overexpressing dynamin were generated as described (19).

Sequestration of β2-ARs—The β2-AR sequestration was quantified as loss of cell surface receptors determined by immunofluorescence flow cytometry as described (5).

Plasmid Construction and Site-specific Mutagenesis—To generate single tyrosine mutants (Y231F and Y597F), tyrosine 231 or tyrosine 597 of rat dynamin I was mutated to phenylalanine by overlapping polymerase chain reaction (UAC(Y) → UUC(F)) (20). The double tyrosine mutant (Y231F/Y597F) dynamin was constructed by recombination of the two single tyrosine mutant constructs.

GST-Grb2 Fusion Proteins—Isolation of total cellular dynamin for analysis of tyrosine phosphorylation was performed by affinity purification with full-length GST-Grb2 fusion protein according to the method of Gout et al. (11). The cDNA encoding GST-Grb2 fusion protein was generously provided by A. R. Saltiel. GST fusion proteins were prepared as glutathione-agarose conjugates as described previously (6, 21). Complexed proteins were dissociated from agarose beads with...
**MDM-polyacrylamide gel electrophoresis sample buffer.**

**Protein Cross-linking**—For the detection of transient, agonist-promoted association of dynamin and c-Src, covalent protein cross-linking with Dithiothreitol (sodium) (DSP, Pierce) was employed. Cells were stimulated in 4.6 ml of phosphate-buffered saline containing 10 mM Hepes, pH 7.4. Stimulation was terminated by the addition of 0.4 ml of 2.5 mM DSP in Me2SO, and plates were rocked for 30 min at room temperature. Reactions were quenched by the addition of 0.1 ml of 1 M Tris, pH 7.5, followed by two washes with ice-cold phosphate-buffered saline/Hepes to remove unreacted DSP. Cell pellets were lysed in RIPA buffer (5, 6) to immunoprecipitation of c-Src-dynamin complexes. 

**Immunoprecipitation and Immunoblotting**—Cell lysates in RIPA buffer were incubated with the specified antibodies and protein AG-Sepharose beads for 2 h at 4°C. Rabbit polyclonal anti-Src antibody (Src-2, Santa Cruz Biotech, Inc.) was used for c-Src immunoprecipitation after protein cross-linking. Dynamin proteins were immunoprecipitated without cross-linking using either mouse monoclonal anti-dynamin antibody (Hud-1, Upstate Biotech, Inc.) or rabbit polyclonal anti-dynamin I-specific antibody (kind gift of S. L. Schmid). Immunoprecipitates or GST-Grb2 fusion complexed proteins were resolved on acrylamide gels, transferred to nitrocellulose filters, immunoblotted with anti-dynamin or anti-phosphotyrosine antibodies (RC20H or PY20H, Transduction Laboratories), and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). SDS-polyacrylamide gel electrophoresis, and the dynamin band was excised from the gel and subsequently used for microsequencing after tryptic digestion (22).

**MAP Kinase Assay**—HA-Erk2 was immunoprecipitated from HEK293 lysates using 6 μg of 12CA5 antibody. Immunoprecipitates were washed three times with RIPA buffer and twice with kinase buffer (23). In vitro kinase reactions using myelin basic protein (MBP) as substrate were performed as described (23). Labeled MBP was quantified using a Storm PhosphorImager (Molecular Dynamics).

**RESULTS AND DISCUSSION**

Spatial distribution and trafficking of receptors control their function. For example, mitogenic signaling emanating from certain GPCRs and RTKs is dependent on receptor internalization (5, 6, 24–26). To determine whether tyrosine kinase activity is required for G protein-coupled receptor endocytosis, isoproterenol-induced internalization of the β2-AR was examined in HEK293 cells pretreated with tyrosine kinase inhibitors. Fig. 1A shows that pretreatment of cells with the generic tyrosine kinase inhibitor herbimycin A reduced isoproterenol-mediated internalization of the β2-AR by 60%. More dramatically, acute exposure of cells to the specific c-Src kinase inhibitor PP1 reduced the isoproterenol-induced β2-AR sequestration by approximately 80% (Fig. 1A). As shown in Fig. 1B, expressing either a c-Src kinase inhibitor (Csk) or a kinase defective, dominant inhibitory form of c-Src (K298M) impaired the isoproterenol-mediated internalization of the β2-AR. Conversely, expression of an activated form of Src (v-Src) increased agonist-dependent sequestration of the β2-AR (Fig. 1B). These results suggest that Src tyrosine kinase activity is required for agonist-dependent internalization of the β2-AR.

A direct interaction between c-Src and dynamin in neuronal cells has been described (27). Because c-Src kinase activity was required for the agonist-mediated internalization of β2-AR, we examined whether dynamin is a phosphotyrosyl protein and a substrate for c-Src. Cellular dynamin was isolated using a GST-Grb2 affinity purification following agonist exposure. As shown in Fig. 2A, isoproterenol stimulation resulted in 2−2.5-fold increase in tyrosine phosphorylation of dynamin (upper immunoblot), which was maximal within 2 min of agonist exposure. These results demonstrate that β2-AR activation induces the rapid tyrosine phosphorylation of dynamin.

Next, we examined whether c-Src is the kinase responsible for the tyrosine phosphorylation of dynamin following activation of the β2-AR. We expressed the c-Src inhibitors, Csk and K298M c-Src, or the constitutively active v-Src and examined their effect on isoproterenol-induced tyrosine phosphorylation of dynamin in HEK293 cells stably expressing dynamin I. Fig. 2B shows that expression of Csk or K298M c-Src inhibited the agonist-induced tyrosine phosphorylation of dynamin, whereas expression of activated Src (v-Src) further enhanced the level of tyrosine phosphorylation of dynamin (upper immunoblot). Thus, agonist stimulation resulted in a net increase in the phosphotyrosine content of the cellular dynamin pool, which could be reversed by inhibition of Src activity. These data mirror the effects of these reagents on β2-AR sequestration (Fig. 1B), suggesting that c-Src-regulated internalization of the β2-AR may be mediated by the c-Src-induced tyrosine phosphorylation of dynamin.

Foster-Barber and Bishop (27) have reported that the purified SH3 domain of c-Src binds dynamin in vitro and that dynamin co-purifies with c-Src from PC12 cell lysates. These authors did not describe tyrosine phosphorylation of dynamin or modulation of c-Src-dynamin complex formation following receptor activation. To test the hypothesis that the agonist-regulated association of dynamin and c-Src might account for the agonist-promoted increase in dynamin tyrosine phosphorylation, we examined the ability of c-Src to form a complex with dynamin following β2-AR activation. Protein cross-linking was used to stabilize potentially transient, agonist-mediated, dynamin-c-Src interactions. HEK293 cells stably expressing dynamin I were treated with or without isoproterenol for 2 min followed by covalent protein cross-linking with DSP. As shown in Fig. 2C, treatment of cells with isoproterenol increased the amount of tyrosine phosphorylated dynamin present in c-Src immunoprecipitates.

Reverse phase high pressure liquid chromatography analysis of trypsin-digested phosphodynamin isolated from isoproterenol-stimulated cells revealed the presence of two major phosphopeptides. Microsequencing and amino acid sequence analyses (19, 22) of one peptide revealed that Tyr<sup>597</sup> of phosphodynamin (Fig. 3A) is phosphorylated (Fig. 3A). Sequence analysis of the second phosphopeptide revealed sequences just N-terminal to a GYG motif (Tyr<sup>231</sup>) that displays high sequence homology to consensus c-Src phosphorylation sites (28).

To determine whether tyrosine phosphorylation of dynamin affected its function, each of these tyrosine residues (at positions 231 and 597) was mutated to phenylalanine singly and in
combination. As shown in Fig. 3 (B and C), whereas isoproterenol treatment increased the tyrosine phosphorylation content of wild-type dynamin, the single tyrosine mutants (Y231F and Y597F), and the double tyrosine mutant (Y231F/Y597F) of dynamin exhibited diminished tyrosine phosphorylation in both isoproterenol-stimulated and nonstimulated cells. The dramatic decrease in tyrosine phosphorylation of Y231F/Y597F mutant strongly suggests that these residues represent the phosphorylated sites in vivo.

In agreement with previous results (5, 15), overexpression of

FIG. 3. Identification of the agonist-induced phosphotyrosyl residues in dynamin. A, schematic representation of dynamin structure and the identification of two putative phosphorylated tyrosine residues. PH, pleckstrin homology domain; GED, GTPase effector domain; PH/PRD, proline/arginine-rich domain. B, effect of isoproterenol on the phosphotyrosyl content of dynamin. Wild type (WT), single (Y231F and Y597F), or double tyrosine mutants (Y231F/Y597F) of dynamin I cDNAs were transiently cotransfected with c-Src into HEK293 cells. Serum-deprived cells were stimulated with 10 μM isoproterenol for 2 min at 37 °C. Dynamin immunoprecipitates were tested for their tyrosine phosphorylation level by immunoblotting with anti-phosphotyrosyl antibodies. Values shown are expressed as fold change in tyrosine phosphorylation following β2-AR stimulation. Data shown represent means ± S.E. from three independent experiments. C, a representative immunoblot showing the effects of isoproterenol on tyrosine phosphorylation levels of wild-type and mutant dynamin proteins. Note the two contemporaneous exposures of the filter (upper and middle panels) to illustrate the effect of mutating the tyrosine residues on phosphotyrosyl content of dynamin. The lower panel shows equivalent loading of dynamin in each lane. IB, immunoblot; IP, immunoprecipitation.

FIG. 4. Effects of tyrosine phosphorylation on the function of dynamin. HEK293 cells were transiently transfected with plasmids expressing the following: empty pRK5 vector (CN), wild-type dynamin I (WT), or each of K44A, Y231F, Y597F, and Y231F/Y597F mutant dynamin I. A, effect on receptor sequestration. Flag-tagged β2-AR was co-transfected with the indicated dynamin cDNAs, and the amount of internalized receptor was measured as described for Fig. 1. Values shown are obtained from five independent experiments done in triplicate and expressed as the percentage of loss of agonist-induced cell surface receptor over unstimulated cells. B and C, isoproterenol-induced activation of MAP kinases. HEK293 cells expressing HA-Erk2, c-Src, and dynamin were stimulated with isoproterenol for 5 min after overnight serum starvation. In vitro kinase assays were performed on HA-Erk2 immunoprecipitates using MBP as substrate. Phosphorylated MBP was visualized by autoradiography, and HA-Erk2 was immunoblotted in parallel using anti-Erk2 antibody. Labeled MBP was quantified using a PhosphorImager. Values are shown as agonist-induced fold increase over unstimulated cells. Data represent means ± S.E. from five independent experiments.
wild-type dynamin did not enhance agonist-promoted sequestration of the β2-AR (Fig. 4A). However, expression of Y231F or Y597F dynamin resulted in a modest reduction of agonist-induced β2-AR internalization. Expression of the Y231F/Y597F dynamin inhibited approximately 70% of the agonist-induced internalization of the β2-AR, which was equivalent to the degree of inhibition observed with GTPase-deficient dynamin K44A. These data suggest that tyrosine phosphorylation of dynamin plays an important role in its ability to support endocytosis.

Recent data suggest that agonist-promoted endocytosis plays a dual regulatory role in signaling pathways emanating from GPCRs or RTKs. Activation of the Erk1/2 MAP kinase pathway by GPCRs, such as those for isoproterenol, lysophosphatidic acid, thrombin, and bombesin (5, 6), as well as RTKs, such as those for EGF (25), nerve growth factor (29, 30), and insulin-like growth factor-1 (26), is dependent on receptor internalization. In the case of the β2-AR, protein kinase A-mediated phosphorylation of the receptor confers receptor-G coupling, with subsequent Ras-dependent Erk1/2 activation mediated by Gβγ-subunits derived from pertussis toxin-sensitive G proteins (31).

In addition, expression of dominant negative mutants of β-arrestin 1 or dynamin, which attenuate agonist-mediated endocytosis, blocks β2-AR-mediated Erk1/2 activation (5). Considerring the role of dynamin in endocytosis, we investigated the effects of expressing wild-type or mutant dynamin on the β2-AR-mediated activation of the Erk2 MAP kinase. Expression of the Y231F or Y597F mutated forms of dynamin reduced the isoproterenol-mediated activation of Erk2 approximately 60% (Fig. 4, B and C). However, expression of the Y231F/Y597F mutant reduced the agonist-induced Erk2 activation by 80–90%, similar to the effects observed using the K44A dynamin (Fig. 4, B and C). These data suggest that c-Src-mediated tyrosine phosphorylation of dynamin is required for Ras-mediated activation of MAP kinase by β2-AR by virtue of its involvement in receptor sequestration.

Our results establish that agonist-induced tyrosine phosphorylation of dynamin is required for β2-AR internalization and internalization-dependent signaling to MAP kinase. Clathrin-coated vesicle-mediated receptor internalization is regulated by the enzymatic (GTPase) activity of dynamin. As shown in Fig. 3A, Tyr231 and Tyr597 reside in the GTPase and pleckstrin homology (PH) domains of dynamin, respectively. Intermolecular interaction between GTPase and GED domains of adjacent dynamins has been reported to regulate the GTPase activity of the enzyme (13). Thus, phosphorylation of Tyr231 might regulate the GTPase activity of dynamin by controlling these intermolecular interactions. Another mutation that disables the GTPase activity of dynamin (K44A) also ablates the ability of dynamin to function in internalization of receptors (14, 15). PH domains in proteins such as phospholipase C-δ1 (32) have been shown to mediate interactions with acidic phospholipids. Although there is as yet no direct evidence for dynamin PH domain-phospholipid interaction, acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate have been shown to stimulate GTPase activity of dynamin in vitro (10, 33). Additionally, G protein βγ subunits, which interact with PH domains of many proteins (34), have been shown to control the GTPase activity of dynamin (33). Tyr231 and Tyr597 are located in one of three variable loops of the dynamin PH domain that possess positive electrostatic potential (35). Phosphorylation of Tyr231 may alter the ability of dynamin to interact with effector molecules such as phosphatidylinositol 4,5-bisphosphate and G protein βγ subunits, resulting in modulation of dynamin GTPase activity. Although the molecular mechanism(s) whereby tyrosine phosphorylation of dynamin regulates its function remain unclear, the present results establish that the c-Src-mediated tyrosine phosphorylation of dynamin provides one mechanism by which GPCRs regulate their own internalization and MAP kinase signaling.