Distinct Roles for Src Tyrosine Kinase in β2-Adrenergic Receptor Signaling to MAPK and in Receptor Internalization*

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G protein-coupled receptors form the largest family of membrane receptors and transmit diverse ligand signals to modulate various cellular responses. After activation by their ligands, some of these G protein-coupled receptors are desensitized, internalized (endocytosed), and down-regulated (degraded). In HEK 293 cells, the Gα-coupled β2-AR adrenergic receptor was postulated to initiate a second wave of signaling, such as the activation of the mitogen-activated protein kinase (MAPK) pathway after the receptor is internalized. The tyrosine kinase c-Src plays a critical role in these events. Here we used mouse embryonic fibroblast (MEF) cells deficient in Src family tyrosine kinases to examine the role of Src in β2-AR adrenergic receptor signaling to the MAPK pathway and in receptor internalization. We found that in Src-deficient cells the β2-AR adrenergic receptor could activate the MAPK pathway. However, the internalization of β2-AR adrenergic receptors was blocked in Src-deficient MEF cells. Furthermore, we observed that in MEF cells deficient in β-arrestin 2 the internalization of the β2-AR adrenergic receptor pathway by the β2-AR adrenergic receptor was normal. Our data demonstrate that although Src and β-arrestin 2 play essential roles in β2-AR adrenergic receptor internalization, they are not required for the activation of the MAPK pathway by the β2-AR adrenergic receptor. In other words, our finding suggests that receptor internalization is not required for β2-AR adrenergic receptor signaling to the MAPK pathway in MEF cells.

G protein-coupled receptors (GPCRs)1 mediate transmembrane signaling for a large number of ligands including hormones, neurotransmitters, photons, odorants, pheromones, chemokines, and other stimuli (1–3). These receptors relay the signals to heterotrimeric G proteins, which directly modulate the activity of enzymes or other effector molecules. One of the prototypes of GPCRs is the β2-adrenergic receptor (β2-AR) that transduces signals from catecholamines, norepinephrine, and epinephrine to the Gα protein, which in turn activates downstream effectors (2). After agonist activation, β2-AR is phosphorylated by GPCR kinases with the subsequent binding of β-arrestin 2 to the phosphorylated β2-AR leading to the internalization (endocytosis) through clathrin-coated pits (4, 5).

GPCRs can relay ligand signals to various cellular signaling pathways. One of these cellular pathways is the mitogen-activated protein kinase (MAPK) pathway (6). The MAPK cascade, an evolutionarily conserved signaling module, stimulates numerous physiological responses including cell growth and differentiation (7). The pathway consists of a MAP kinase kinase that phosphorylates and activates a MAP kinase kinase, which in turn phosphorylates the TXY activation loop of MAP kinase (8). The first characterized subfamily of MAP kinases, termed extracellular signal-regulated kinases (ERKs), and is activated by growth factor receptors, G protein-coupled receptors, and other types of receptors. The most common MAPK pathway involves Ras/Raf kinase (a MAP kinase kinase kinase), and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (a MAP kinase kinase) (9).

GPCRs seem to activate the ERK MAPK pathway by diverse signaling pathways depending on the receptors and the cell types. Some of these activation pathways from GPCRs to ERK MAPK involve tyrosine kinases (10–16). For example, previously we showed that Src family tyrosine kinases are essential for Gαi signaling to ERK MAPK in DT40 chicken B lymphoma cells (11). In other GPCR signaling to ERK MAPK or in other cell types, tyrosine kinases might not be essential. We have shown that in S49 mouse T lymphoma cells, Gαi signaling to the ERK MAPK pathway is independent of Src family tyrosine kinases. Instead, a protein kinase A-dependent pathway was used (17, 18). In HEK 293 cells, it was reported that isoproterenol stimulation of β2-AR leading to the activation of ERK MAPK requires Src and receptor internalization (19).

Src family tyrosine kinases are a major group of cellular signal transducers (20). c-Src was the first identified protein tyrosine kinase (21, 22). These tyrosine kinases can be activated by various extracellular signals and modulate a variety of cellular functions including proliferation, survival, adhesion, and migration (20). GPCRs have been shown to stimulate tyrosine phosphorylation of cellular proteins (20). Various G protein-mediated physiological functions are sensitive to tyrosine kinase inhibitors. Many GPCRs are able to increase the activity of Src family tyrosine kinases (10–13, 15, 16, 23–27). Although the mechanism by which other G proteins activate Src family tyrosine kinases is not known, we have shown previously that Gαi and Gαq can directly interact with and activate Src (18, 28).

During our investigation of the role of tyrosine kinases in G protein signaling, we notice that in mouse embryonic fibroblast (MEF) cells deficient in Src family tyrosine kinases (SYF cells) a constitutively active mutant of Gαq (GαqQ227L) is able to increase the kinase activity of ERK MAPK. Indeed stimulation of the endogenous Gα-coupled β2-AR also activates ERK MAPK.

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1 The abbreviations used are: GPCR, G protein-coupled receptor; β2-AR, β2-adrenergic receptor; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEF, mouse embryonic fibroblast; GFP, green fluorescent protein.

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in SYF cells. However, the internalization of β2-AR is blocked in SYF cells. Thus, these observations implicate that receptor internalization might not be necessary for the activation of the ERK MAPK pathway by β2-AR. To confirm this, we used MEF cells deficient in β-arrestin 2 because β-arrestin 2 has been shown to be essential for β2-AR internalization (29). We found that although β-arrestin 2 deficiency impairs β2-AR internalization, it does not affect β2-AR signaling to the ERK MAPK pathway. Together, these data demonstrate that Src plays different roles in β2-AR signaling to MAPK and in receptor internalization and that receptor internalization is not required for β2-AR signaling to ERK MAPK in MEF cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmid Constructs—The MEF cells deficient in Src family tyrosine kinases (SYF cells) were purchased from ATCC. The SYF/Src cells were established by stably transfecting human c-Src into SYF cells (28). The MEF cells deficient in either β-arrestin 1 or deficient in β-arrestin 1 and 2 are kindly provided by Dr. R. Lefkowitz (Duke University) (29). All plasmid cDNAs for human G proteins were purchased from Guthrie Research Institute. The cDNA for the human β2-AR was kindly provided by Dr. C. Malbon (State University of New York at Stony Brook) and subcloned into the pcDNA3 vector. A GFP tag was inserted into the′ ′end of β2-AR.

ERK MAPK Assay—The p44/42 MAP kinase assay was performed using kits from Cell Signaling Technology as described previously (11). Whole cell lysates were prepared from MEF, SYF, SYF/Src, β-arrestin 2−/−, or β-arrestin 1−/−2−/− fibroblast cells. Cells were either treated or not with isoproterenol (10 μM) for 5 min. A monoclonal antibody to the phospho-p44/42 ERK MAPK (cross-linked to agarose beads) was added to immunoprecipitate the active ERK MAPK from cell lysates. Substrates (200 μM ATP and 2 μg glutathione-S-transferase-Elk-1 fusion protein) were added, and the reaction was allowed to proceed at 30 °C for 30 min. After SDS-PAGE, the ERK MAPK activity (the phosphorylation of glutathione S-transferase-Elk-1 by ERK MAPK) was measured by Western blotting with an anti-phospho-Elk-1 antibody (Cell Signaling Technology).

Receptor Internalization—Cells were transiently transfected with a plasmid carrying a GFP-tagged human β2-adrenergic receptor (pcDNA3-β2-AR-GFP). Twenty-four h after transfection, cells were split onto poly-d-lysine-coated glass coverslips. Forty-eight h after transfection, cells were treated with or without 10 μM isoproterenol at 37 °C for 30 min, rinsed quickly three times with phosphate-buffered saline, and further incubated in 3.7% formaldehyde for 20 min at room temperature. After being rinsed again three times in phosphate-buffered saline, the coverslips were mounted on a microscope slide with Vecta Shield mounting medium (Vector Laboratories) before imaging by fluorescence microscopy. Fluorescence microscopy was performed on a Zeiss Axiosvert 35 microscope.

RESULTS

Src Family Tyrosine Kinases Are Not Required for Goα2Q227L Stimulation of ERK MAPK in MEF Cells—To genetically test the role of Src family tyrosine kinases in Gs signaling to the ERK MAPK pathway, we investigated the stimulation of ERK MAPK by the constitutively active mutant of Goα (Goα-Q227L) in Src family tyrosine kinase knock-out MEF cells. We used the SYF cells that were derived from Src, Yes, and Fyn triple knock-out mouse embryos (30). Because Src, Yes, and Fyn are the three ubiquitously expressed members of the Src family tyrosine kinases, no Src family tyrosine kinase activity was detected in these SYF cells (30). We found that transient expression of Goα-Q227L in the control wild-type MEF cells led to increased activity of ERK MAPK (Fig. IA). In SYF cells, expression of Goα-Q227L also increased the ERK MAPK activity to a similar degree (Fig. IB). These genetic data demonstrated that Src family tyrosine kinases are not required for Goα-Q227L signaling to the ERK MAPK pathway. As controls, we also examined the requirement for Src family tyrosine kinases by some other G proteins signaling to ERK MAPK in MEF cells. As shown in Fig. 1C, transient expression of constitutively active mutants of Goα12 (Goα12-Q231L), Goα13 (Goα13-Q226L), Goα4 (Goα4-Q209L), Goα9 (Goα9-Q205L), and Goβ1Y2 led to the activation of ERK MAPK to different degrees. Interestingly, in SYF cells, except for Goα13-Q226L, the other G protein mutants (Goα-Q205L, Goα12-Q231L, Goα4-Q209L, and Goβ1Y2) failed to stimulate ERK MAPK (Fig. 1D). These data suggest that Goα13-Q226L does not require Src family tyrosine kinases signaling to the ERK MAPK pathway in MEF cells, whereas Goα-Q205L, Goα12-Q231L, Goα-Q209L, and Goβ1Y2 require Src family tyrosine kinases for the activation of ERK MAPK in MEF cells. In this work, we focused on the Gs signaling to ERK MAPK. Thus, we did not examine further the Src-dependent signaling by other G proteins to the ERK MAPK pathway in MEF cells.

Src Family Tyrosine Kinases Are Not Required for β2-Adrenergic Receptor Stimulation of ERK MAPK in MEF Cells—The above study examined the role of Src family tyrosine kinases in signaling to the ERK MAPK pathway by exogenously expressing constitutively active Gs mutant proteins. Next we examined the requirement of endogenous Gs protein-coupled receptors for Src family tyrosine kinases. We used the β2-adrenergic receptor as a representative for Gs-coupled receptors. Treatment of
MEF cells with the β2-AR agonist isoproterenol increased the cAMP levels in MEF and SYF cells, indicating the presence of endogenous β2-adrenergic receptors in these cells (Fig. 2A). As shown in Fig. 2B, treatment of SYF cells with isoproterenol led to an increased activity of ERK MAPK, indicating that Src family tyrosine kinases are not essential for β2-AR signaling to ERK MAPK. Isoproterenol also increased the activity of ERK MAPK in SYF/Src cells (expression of Src in SYF cells) (Fig. 2B). Similar results also were observed with another β2-AR agonist, terbutaline (data not shown). This is consistent with the above data that showed that Gαo-mediated activation of ERK MAPK is independent of Src family tyrosine kinases. Hence, the endogenous β2-AR still was able to stimulate ERK MAPK in SYF cells.

β-Arrestin 2 Is Required for β2-Adrenergic Receptor Internalization—It has been reported that β2-AR internalization might initiate a second wave of intracellular signaling, including the activation of the ERK MAPK pathway in HEK 293 cells (19). To investigate whether that is the case in MEF cells, we genetically examined the role of β-arrestin proteins in β2-AR stimulation of ERK MAPK. Radioligand binding studies with β-arrestin 2−/−, β-arrestin 1−/−, and β-arrestin 1−/− 2−/− MEF cells showed that the internalization of β2-AR in β-arrestin 2−/− cells and β-arrestin 1−/− 2−/− cells was significantly impaired (29). On the other hand, the β2-AR internalization was normal in β-arrestin 1−/− cells (29). In the control wild-type MEF cells, transient expression of a C-terminal GFP-tagged β2-AR showed membrane staining as well as cytoplasmic staining (Fig. 3A). Thirty min after the addition of isoproterenol to these cells, an increased level of internalization of these GFP-tagged β2-adrenergic receptors was observed (Fig. 3B). On the other hand, in β-arrestin 2−/− cells, treatment with isoproterenol did not lead to internalization of the GFP-tagged β2-ARs. These results are consistent with the previous report that β-arrestin 2 plays a critical role in β2-AR internalization (29) (Fig. 3, C and D).

Next we investigated the potential role of β-arrestin proteins and thus receptor internalization in β2-AR-initiated activation of ERK MAPK. As shown in Fig. 4, addition of isoproterenol to the β-arrestin 1−/− 2−/− cells led to increased ERK MAPK activity (same as in the wild-type MEF cells and β-arrestin 2−/− cells) (Fig. 4). Moreover, similar results also were observed with another β2-AR agonist, terbutaline (data not shown). Together, these results demonstrate that in MEF cells, β-arrestin proteins and thus receptor internalization are not essential for β2-AR signaling to the ERK MAPK pathway.

**Src Family Tyrosine Kinases Are Required for β2-Adrenergic Receptor Internalization**—Because we did not find an essential role for receptor internalization in the activation of the ERK MAPK pathway by β2-AR in MEF cells and because Src has been reported to form a complex with β-arrestin 2, we examined the genetic requirement for Src family tyrosine kinases in β2-AR internalization. In contrast to what was observed in the control wild-type MEF cells (Fig. 3, A and B), isoproterenol did not induce the internalization of the GFP-tagged β2-AR in SYF cells (Fig. 5, A and B). To confirm that this defect in receptor internalization was caused by the absence of Src family tyrosine kinases, we re-introduced Src into SYF cells. Re-expression of Src in these SYF cells rescued the receptor internalization phenotype (Fig. 5, C and D). These data indicate that even though Src family tyrosine kinases are not required for β2-AR signaling to the ERK MAPK pathway they are essential for β2-AR internalization. Therefore, Src family tyrosine kinases play distinct roles in β2-AR signaling to ERK MAPK and in receptor internalization.

**DISCUSSION**

**Role of Src Family Tyrosine Kinases in β2-Adrenergic Receptor Signaling to ERK MAPK**—Although prolonged exposure to cAMP leads to the inhibition of ERK MAPK in some types of cells, ligand stimulation of β2-AR is able to transiently stimulate ERK MAPK in most reported cases (17, 31–40). In S49 mouse lymphoma cells, we previously reported a Gαi2-adenyl cyclase-cAMP-protein kinase A-dependent pathway leading to ERK MAPK activation (17). This classical Gαi2-protein kinase A signaling pathway also has been implicated in activating the ERK MAPK pathway through β2-AR stimulation in other cells such as the HEK 293 cells (38). Furthermore, this Gαi signaling pathway via protein kinase A to ERK MAPK also has been reported for other Gαi-coupled receptors (36, 41). Because this pathway is independent of Ras, it is different from the growth factor receptor tyrosine kinase signaling to the ERK MAPK pathway.

However, there are disagreements on whether Src family tyrosine kinases are involved in β2-AR signaling to the ERK MAPK pathway. In HEK 293 cells, β-arrestin was reported to form a complex with Src and brought Src to the β2-AR, leading to the receptor desensitization/internalization process, which initiates a second wave of signaling including the ERK MAPK pathway (19). On the other hand, we show here that in MEF cells Src is not essential for β2-AR stimulation of ERK MAPK. Moreover, treatment of HEK 293 cells with Src family kinase inhibitors did not block Gα12-adenosine receptor signaling to the ERK MAPK pathway (42). The different roles of Src in β2-AR stimulation of ERK MAPK in these reports might reflect either the different cell types used or the methods used.

Interestingly, we noticed that the basal activity of ERK MAPK in SYF cells was consistently higher than in MEF cells and SYF/Src cells (Fig. 2B; also Fig. 1, A–D). This difference in basal activity of ERK MAPK is not caused by a difference in ERK MAPK protein expression levels in MEF and SYF cells. These data imply that the basal ERK MAPK activity in MEF cells was suppressed by c-Src. Inhibition of ERK by activated c-Src was reported recently in fibroblast cells (43). However, because MEF cells were serum-starved, c-Src was likely in the down-regulated (inactive) state. Thus, it seems that the down-regulated c-Src has an unexpected function here. This might be reminiscent of the MAPK Kss1 in the yeast *Saccharomyces cerevisiae*. Kss1 in its inactive form is a potent negative regulator of invasive growth (44, 45). The nature of this potential
negative regulation of basal ERK MAPK by c-Src needs further investigation.

Role of Src Family Tyrosine Kinases in \( \beta_2 \)-Adrenergic Receptor Internalization—For \( \beta_2 \)-AR internalization, ligand stimulation of \( \beta_2 \)-AR leads to the activation of G protein-coupled receptor kinases and the phosphorylation of some C-terminal Ser/Thr residues of \( \beta_2 \)-AR by G protein-coupled receptor kinases. This phosphorylation in turn promotes the association of \( \beta_2 \)-AR with \( \beta_2 \)-arrestin 2 and subsequent internalization (4, 5). The essential role of \( \beta_2 \)-arrestin 2 in \( \beta_2 \)-AR internalization was confirmed in MEF cells from \( \beta_2 \)-arrestin 2 knock-out mice (29). \( \beta_2 \)-Arrestins can interact directly with clathrin (46), which could promote receptor internalization through clathrin-coated pits (4, 5).

Following the above \( \beta_2 \)-AR internalization scheme, it was surprising to see the inhibition of \( \beta_2 \)-AR internalization in SYF cells. Our data strongly indicate that Src family tyrosine kinases are involved in the \( \beta_2 \)-AR internalization process. There was some suggestive evidence that Src family tyrosine kinases might act upstream of G protein-coupled receptor kinases in \( \beta_2 \)-AR internalization. First, overexpression of a dominant-negative mutant of c-Src, treatment with PP2 inhibitor, and antisense oligodeoxynucleotides in human epidermoid A431 carcinoma cells inhibited ligand-induced \( \beta_2 \)-AR internalization (47). It was proposed that ligand stimulation of \( \beta_2 \)-AR leads to the phosphorylation of tyrosine residue 350 at the C-terminal tail of the \( \beta_2 \)-AR by a yet to be identified tyrosine kinase. Src then uses its Src homology 2 domain to bind this phosphorylated tyrosine 350. This association to the \( \beta_2 \)-AR also activates Src (47). Second, it was reported that Src might phosphorylate and activate G protein-coupled receptor kinases (48). The detailed mechanism by which Src family tyrosine kinases participate in the \( \beta_2 \)-AR internalization needs further investigation (49). Nevertheless, our data clearly show that Src is essential for \( \beta_2 \)-AR internalization in MEF cells.

Role of Receptor Internalization in \( \beta_2 \)-Adrenergic Receptor Signaling to ERK MAPK—Previous studies that used a dominant-negative dynamin mutant and/or mutant arrestin proteins to examine the potential role of receptor internalization in the \( \beta_2 \)-AR signaling to ERK MAPK yielded inconsistent results. In HEK 293 cells and COS-7 cells, although some researchers reported that receptor internalization was required for \( \beta_2 \)-AR initiated activation of the ERK MAPK pathway (19, 50, 51),
others reported no requirement for receptor internalization in β2-AR signaling to ERK MAPK (40, 52). Our genetic data clearly demonstrate that the receptor internalization is not essential for β2-AR signaling to ERK MAPK in MEF cells.

For GPCRs as a whole, whether the receptor internalization is needed for the activation of the ERK MAPK pathway remains to be fully clarified. Some researchers suggested that receptor internalization seems to be needed for activation of the ERK MAPK pathway by some GPCRs (51, 53, 54), whereas others reported no requirement for receptor internalization in β2-AR signaling to ERK MAPK (40, 52). Our genetic data clearly demonstrate that the receptor internalization is not essential for β2-AR signaling to ERK MAPK in MEF cells.

In summary, we have shown that Src family tyrosine kinases are not required for GoαQ227L stimulation of ERK MAPK in MEF cells. Ligand stimulation of the endogenous Goα-coupled β2-AR also activates ERK MAPK in SYF cells, whereas the internalization of β2-AR is blocked in SYF cells. Furthermore, we found that although the β-arrestin 2 deficiency impairs β2-AR internalization, it has no effect on β2-AR signaling to the ERK MAPK pathway. Therefore, Src and β-arrestin 2 play different roles in β2-AR signaling to ERK MAPK and in receptor internalization, and receptor internalization is not required for β2-AR signaling to ERK MAPK in MEF cells.

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