Direct Binding of Activated c-Src to the β3-Adrenergic Receptor Is Required for MAP Kinase Activation

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Both β₂- and β₃-adrenergic receptors (ARs) are able to activate the extracellular signal-regulated kinase (ERK) pathway. We previously showed that c-Src is required for ERK activation by β₂AR and that it is recruited to activated β₂AR through binding of the Src homology 3 (SH3) domain to proline-rich regions of the adapter protein β-arrestin1. Despite the absence of sites for phosphorylation and β-arrestin binding, ERK activation by β₂AR still requires c-Src. Agonist activation of β₂AR, but not β₃AR, led to redistribution of green fluorescent protein-tagged β-arrestin to the plasma membrane. In β-arrestin-deficient COS-7 cells, β-agonist-dependent co-precipitation of c-Src with the β₂AR required exogenous β-arrestin, but activated β₃AR co-precipitated c-Src in the absence or presence of β-arrestin. ERK activation and Src co-precipitation with β₃AR also occurred in adipocytes in an agonist-dependent and pertussis toxin-sensitive manner. Protein interaction studies show that the β₂AR interacts directly with the SH3 domain of Src through proline-rich motifs (PXXP) in the third intracellular loop and the carboxyl terminus. ERK activation and Src co-precipitation were abolished in cells expressing point mutations in these PXXP motifs. Together, these data describe a novel mechanism of ERK activation by a G protein-coupled receptor in which the intracellular domains directly recruit c-Src.

During the past several years, transmembrane signaling traffic through G protein-coupled receptors (GPCRs) has grown from the classic G protein effectors such as adenylyl cyclase and phospholipases to include novel mechanisms for activation of mitogen-activated protein (MAP) kinase cascades. These signaling systems typically involve receptor and non-receptor tyrosine kinases as scaffolds and intermediaries (1–6). An example of this flexibility in GPCR signaling includes the β₂-adrenergic receptor (β₂AR). Although this receptor is classically known to couple to Gs and stimulate adenylyl cyclase, it can also activate the ERK1/2 MAP kinase pathway (7, 8). In some cell types, the β₂AR activates ERK through its coupling to a PTX-sensitive Gᵢ protein and subsequent Ras-dependent MAP kinase activation (7, 9), whereas in other systems this occurs in a PTX-independent and a Gᵢ and cAMP-dependent process (10, 11) through the activation of Rap1 (11).

In exploring the mechanisms of β₂AR-stimulated MAP kinase activation, we have found that some of the same signaling molecules required for receptor desensitization can also be intimately involved in the activation of the MAP kinase cascade. Following agonist activation, most GPCRs are phosphorylated by GPCR kinases (GRKs), with subsequent binding of β-arrestin to the phosphorylated receptor serving to interdict G protein coupling and signal transduction (5, 12, 13). However, in addition to its role in desensitization, β-arrestin can also participate in the events leading to MAP kinase activation. Binding of β-arrestin1 to the agonist-activated β₂AR rapidly recruits c-Src to the receptor (12, 14). This recruitment appears to be mediated by an interaction between the amino-terminal proline-rich region of β-arrestin1 and the SH3 domain of c-Src (13, 15).

The β₂AR is a member of the βAR subfamily of GPCRs that is expressed predominantly in adipocytes. Because selective β₂AR agonists have been shown to prevent or even reverse obesity and diabetes in various animal models (16–18), increased attention has been focused upon the molecular and physiological regulation of this receptor as a therapeutic target (19). Early studies of β-adrenergic stimulation of adenylyl cyclase in adipocytes by Rodbell and colleagues (20) indicated the presence of a PTX-sensitive component. In examining this issue, we showed that this effect is due to the presence of the adipoocyte-specific β₂AR and its ability to simultaneously couple to both Gᵢ and Gₛ, leading to the activation of the cAMP-dependent protein kinase A and ERK1/2 pathways, respectively (9). Because GRK-mediated phosphorylation is necessary for β-arrestin binding (reviewed in Ref. 21), but the β₂AR lacks sites for phosphorylation (22), we concluded that the β₂AR must employ a novel mechanism of ERK activation. Here, we demonstrate that conserved proline-rich motifs in the third intracellular loop and carboxyl terminus of the β₂AR directly recruit c-Src in a β₂AR agonist- and PTX-sensitive manner. This interaction occurs specifically through the SH3 domain of c-Src. Our findings establish a new mechanism whereby some GPCRs can acquire ligand-induced tyrosine kinase activity by means of direct recruitment of Src kinases.

**Experimental Procedures**

**Chemicals and Construction of Plasmids**—The β₂AR agonist CL316,243 was a gift from American Cyanamid Co. (Pearl River, NY). L-Isoproterenol and propranolol were from Sigma and selective c-Src inhibitor PP2 from Calbiochem. For plasmids Xa-2-Loop3 (Wt), Xa-2-Loop3 (MutIII), and Xa-2-Loop3 (MutII), the wild-type or mutated third intracellular domain (amino acids 222–289) or the carboxyl terminus (amino acids 344–400) of mouse β₂AR were cloned into PinPoint Xa-2 vector at HindIII and BglII sites under the control of the SV40 promoter. All plasmid constructs were verified by sequencing.
GSK fusion proteins of c-Src containing either the SH3 and SH2 domains (GST-SH3/SH2) or the SH2 domain alone (GST-SH2) were prepared as described previously (23).

**Cell Culture and Transfections and Signal Transduction Assays—**C3H10T1/2 (T1/2) preadipocytes and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in 6-well dishes as detailed (13, 24). Cells were transfected with HA-β2AR DNA (2 μg for T1/2 cells; 1 μg for COS-7 cells) and 5 μl of LipofectAMINE (Life Technologies, Inc.). For T1/2, on day 2 cells were induced to differentiate (1 μM rosiglitazone, 0.1 μM LGD1069, 200 nm insulin).

Prior to each analysis, the density of receptor per cell was assayed by fluorescence-activated cell sorting. MAP kinase assays were performed as previously described (9). MAP kinase was migrated as determined by its mobility on a 4–20% SDS acrylamide gradient gel electrophoresis (Novagen), transferred to nitrocellulose membranes, and identified by staining with streptavidin-conjugated alkaline phosphatase. Immunoprecipitations of HA-tagged c-Src and β2AR from intact cells and immunoblotting for co-precipitated proteins were performed as described previously (25).

**RESULTS AND DISCUSSION**

**Activation of the Ras-dependent ERK cascade by many GPCRs requires Src kinase activity (13, 25). For the β2AR, ERK activation depends on the delivery of β-arrestin-bound c-Src to the receptor (13). However, it is unclear whether other GPCRs utilize this same mechanism. As shown in Fig. 1, β2AR-mediated ERK activation similarly requires Src kinase activity, as demonstrated by its concentration-dependent sensitivity to the Src-specific tyrosine kinase inhibitor, PP2. Complete inhibition was achieved within the range of 1 to 5 μM; a concentration previously established to selectively inhibit Src kinase (8, 26). Inhibition of β2AR-mediated ERK activation was also observed when the C-terminal Src kinase was co-expressed with β2AR (data not shown). Because GRK-mediated phosphorylation of receptors is necessary for β-arrestin binding (21), but the β2AR is not phosphorylated by GRKs, we hypothesized that agonist stimulation of β2AR would not lead to β-arrestin binding. This hypothesis is confirmed as illustrated in Fig. 2, which compares the effects of agonist stimulation on the cellular distribution of a chimeric β-arrestin-2-GFP in HEK-293 cells expressing either the human β2AR or the mouse β2AR. Isoproterenol (10 μM) stimulation of the β2AR promotes the rapid translocation of β-arrestin-2-GFP from a diffuse cytosolic distribution to the plasma membrane where it aggregates with the receptor in membrane-associated puncta (14). In contrast, stimulation of cells expressing the mouse β2AR with the selective β2AR agonist CL316,243 (5 μM) fails to induce β-arrestin-2-GFP translocation. Thus, although β2AR-stimulated ERK activation is Src-dependent, similar to the β2AR, the β2AR response occurs without the formation of complexes between β2AR and β-arrestin.

We previously showed that c-Src interacts with proline-containing motifs in the β-arrestin amino terminus and the SH3 domain of c-Src, although the c-Src catalytic domain also contributes significantly to this binding (13). Interestingly, although the β2AR does not recruit β-arrestin, all species homologues of this receptor contain highly conserved proline residues in both the third intracellular domain and the carboxyl terminus that are completely absent from the β2AR. Two of these proline clusters in each domain contain the sequence PXXP, which represents the minimal consensus motif for SH3 domain binding (27–29). We therefore tested the hypothesis that these proline-rich motifs within the β2AR might directly recruit SH3 domain-containing proteins to the receptor, obviating the need for β-arrestin to function as an adapter protein.

First, we determined whether β2AR could directly recruit Src kinases to activate the ERK pathway in the absence or presence of over-expressed β-arrestin in COS-7 cells, which express little endogenous β-arrestin (30). As shown in Fig. 3 (lanes 1–3), agonist treatment resulted in the detectable co-precipitation of c-Src with the β2AR only in the presence of co-expressed β-arrestin. As expected from earlier studies (13), the provision of β-arrestin dramatically enhanced β2AR-mediated ERK phosphorylation under these conditions. In contrast, the expression of β-arrestin had no effect on responses mediated by the β2AR. Robust β2AR agonist-dependent co-precipitation of c-Src and ERK1/2 activation was observed, which did not require the presence of β-arrestin (lanes 4–6). Src that co-precipitated with β2AR and β2AR was also in its activated (dephosphorylated) state.

Second, because the physiological site of expression of the β2AR is the adipocyte, a key question is whether the β2AR directly recruits Src kinase in adipocytes as observed in COS-7 cells. We performed similar co-precipitation experiments in the mouse white adipocyte cell line, C3H10T1/2 (31). As shown in Fig. 4A, by day 4 of differentiation, C3H10T1/2 cells express the adipocyte-specific genes β2AR and the fatty acid-binding protein aP2 (32, 33). Fig. 4B shows that the β2AR-selective agonist CL316,243 is capable of triggering ERK activation in both nontransfected (NT) cells (via the endogenous β2AR) and in the HA-β2AR transfected cells, but ERK activation was abolished by inactivation of Gt with PTX. Fig. 4C shows that, as observed in COS-7 cells, Src kinase co-precipitates with the HA-β2AR in C3H10T1/2 adipocytes, and this interaction is both agonist- and Gt-dependent.
These results taken together indicate that the β3AR can mediate the β-arrestin-independent recruitment of c-Src. To address whether binding between proline-rich motifs in the β3AR and the c-Src SH3 domain might be responsible for this interaction, we first tested whether peptides derived from these regions of the β3AR, as shown in Fig. 5A, would bind to GST-Src fusion proteins in vitro. As shown in Fig. 5B, biotinylated fusion proteins derived from both the third intracellular domain (Loop3) and the carboxyl terminus (Tail) of the wild-type β3AR bound to the GST-Src SH3/SH2 but not to the GST-Src SH2 fusion protein. There was no interaction with GST alone. Consistent with an SH3 domain-mediated interaction, mutants of the β3AR Loop3 (L1) and Tail (T1) peptides in which Ser was substituted for Pro in the PXXP motifs were no longer able to interact with the GST-Src SH3/SH2 peptide. In addition, we found that the wild-type β3AR Loop3 and Tail peptides could precipitate endogenous c-Src and Grb2 from whole cell lysates of HEK-293 cells (not shown). Collectively, these data suggest that proline-rich motifs in both Loop3 and the Tail of the β3AR possess the capacity to bind SH3 domains.

To assess the functional role of the β3AR proline-rich motifs in Src-dependent activation of the ERK cascade, mutant β3ARs were constructed in which these motifs were disrupted by site-directed mutagenesis. Mouse β3ARs containing the L1, L2 (a deletion of Loop3 amino acids Ser-242 to Pro-266), or T1 mutations were expressed in COS-7 cells and assayed for the ability to co-precipitate endogenous c-Src and to induce ERK1/2 phosphorylation and cAMP production. As shown in Fig. 6, mutation or deletion of the PXXP motifs in either the third intracellular loop or the carboxyl terminus resulted in a striking dissociation of β3AR-mediated c-Src binding and ERK activation from β3AR-mediated stimulation of adenyl cyclase. Fig. 6A shows that endogenous c-Src co-precipitated with wild-type β3AR in an agonist-dependent manner, whereas co-precipitation of c-Src with each of the mutant receptors was markedly impaired. Similar effects were observed for β3AR-mediated ERK1/2 activation, which was abolished by the L1, L2, and T1 mutations (Fig. 6C). In contrast, β3AR-mediated production of cAMP was completely unaffected by disruption of the PXXP motifs (Fig. 6E).

These data suggest that the ability of the β3AR to mediate Src-dependent activation of the ERK cascade, but not its ability to interact with Gs protein and stimulate the cAMP pathway, is dependent upon the integrity of the PXXP motifs in the third intracellular domain and the carboxyl terminus of the receptor. Each of these motifs is sufficient to bind to Src-GST SH3 domains in vitro, and site-directed mutagenesis of either motif prevents the agonist-dependent formation of complexes between the β3AR and c-Src when the mutant receptors are expressed in intact cells. The apparent necessity for intact PXXP motifs in both the third intracellular loop and the carboxyl terminus of the β3AR for agonist-dependent Src co-precipitation and functional ERK activation is not clear, but it implies that a novel multimeric complex containing at least activated c-Src and two domains of the β3AR is formed on the receptor itself to trigger this signaling cascade. However, the exact nature of this complex is unknown, and the stoichiometry of the receptor-Src interaction will require further mutagenesis and structural analysis.

The distinct strategies employed by the β2AR and β3AR to recruit Src kinases illustrate the flexibility that characterizes the mechanisms of G protein coupling, agonist-induced receptor sequestration, and activation of tyrosine protein kinases by heptahelical receptors. In the case of the β2AR, G protein coupling and
rates the adapter protein role of β-arrestin within the receptor, thus allowing Src recruitment and ERK activation to proceed independently of receptor sequestration.

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