Direct Binding of the β1 Adrenergic Receptor to the Cyclic AMP-Dependent Guanine Nucleotide Exchange Factor CNrasGEF Leads to Ras Activation

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G-protein-coupled receptors (GPCRs) can indirectly activate Ras primarily through the βγ subunits of G proteins, which recruit c-Src, phosphatidylinositol 3-kinase, and Grb2-SOS. However, a direct interaction between a Ras activator (guanine nucleotide exchange factor [GEF]) and GPCRs that leads to Ras activation has never been demonstrated. We report here a novel mechanism for a direct GPCR-mediated Ras activation. The β1 adrenergic receptor (β1-AR) binds to the PDZ domain of the cyclic AMP (cAMP)-dependent Ras exchange factor, CNrasGEF, via its C-terminal SkV motif. In cells heterologously expressing β1-AR and CNrasGEF, Ras is activated by the β1-AR agonist isoproterenol, and this activation is abolished in β1-AR mutants that cannot bind CNrasGEF or in CNrasGEF mutants lacking the catalytic CDC25 domain or cAMP-binding domain. Moreover, the activation is transduced via Gαi and not via Gβγ. In contrast to β1-AR, the β2-AR neither binds CNrasGEF nor activates Ras via CNrasGEF after agonist stimulation. These results suggest a model whereby the physical interaction between the β1-AR and CNrasGEF facilitates the transduction of Gαi-induced cAMP signal into the activation of Ras. The present study provides the first demonstration of direct physical association between a Ras activator and a GPCR, leading to agonist-induced Ras activation.
tagged wild-type or mutant β1-AR (20 μg) was incubated with GST or GST-PDZ (20 μg) immobilized on glutathione-Sepharose beads in HNTG for 2 h at 4°C. Bound β1-AR was identified by using anti-His antibody (1:2,000). For communoprecipitations, HEK-293T cell lysates expressing either untransfected or transfected HA-β1-AR (500 μg of each) were incubated with anti-HA antibody for 1 h at 4°C, followed by the addition of 30 μl of Protein G-Sepharose for an additional 1 h. After six washes with 1 ml of lysis buffer, the immunoprecipitated proteins were eluted from beads with 1% SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with anti-Flag antibody (1:10,000).

Immunofluorescence confocal microscopy. HEK-293T cells at 48 h posttransfection or primary cultured neurons and coronary artery smooth muscle cells after 7 to 10 days in culture were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and/or permeabilized with 0.25% Triton X-100. Transfected HA-β1-AR expressed in HEK-293T cells was first stained with anti-HA antibody (1:1,000) and then stained with Cy3-conjugated anti-mouse secondary antibody (1:500). Endogenous β1-AR in coronary artery smooth muscle cells was stained with anti-β1-AR antibody (1:200) and Cy3-conjugated anti-rabbit secondary antibody (1:200). Endogenous CNrasGEF in that primary culture was stained with anti-CNrasGEF antibody (1:200) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (1:500). Since both anti-β1-AR and anti-CNrasGEF antibodies are polyclonal, we covalently conjugated the β1-AR antibody to the fluorophore TRITC prior to double-labeling experiments according to the manufacturer’s instructions (Pierce). For double-labeling experiments, primary cortical neurons were first stained with anti-CNrasGEF antibody (1:200), subsequently stained with FITC-conjugated anti-rabbit secondary antibody (1:1,000), and then stained with anti-β1-AR antibody (1:500). The specificity of the staining was tested with either rabbit preimmune serum for CNrasGEF or normal rabbit serum conjugated with TRITC (1:50) for β1-AR. Subcellular localization of proteins was examined with a Zeiss confocal microscope, and the fluorescence intensity was quantified by using the LSM510 ImagePC software.

Ras activation. HEK-293T cells were transfected with the various constructs described in the text, starved overnight, and then subjected to the indicated treatments. Cells were then lysed with Ras lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM NaVO₄, 10 mM MgCl₂, 1 mM EDTA, 10 mg of leupeptin/ml, 10 mg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride), and the level of Ras-GTP was determined by precipitation with a GST fusion protein of the Ras-binding domain on Raf1 (Raf-RBD; Upstate Biotechnology), which recognizes only active, GTP-bound Ras (12). Ras-GTP was detected by immunoblotting with anti-Ras antibodies (Quality Biotech).

cAMP production assay. HEK-293T cells were transfected with appropriate constructs (10⁶ cells/60-mm dish). Intracellular cAMP levels were measured by using a cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

RESULTS

CNrasGEF specifically binds to β1-AR but not to β2-AR. The β adrenergic receptors (β1-AR and β2-AR) are known to elevate cAMP upon agonist stimulation and to possess C-terminal motifs (Ser-x-Val/Leu) that can bind PDZ domains (22, 27). We thus first tested whether these receptors can bind to the PDZ domain of CNrasGEF. Figure 1 shows that GST fusion protein encoding the PDZ domain of CNrasGEF (GST-PDZ) binds to the β1-AR (Fig. 1A) but not to the β2-AR (Fig. 1C) expressed in HEK-293T cells, demonstrating binding specificity toward β1-AR. The PDZ binding motif of β1-AR consists of a C-terminal SKV sequence, whereas that of the β2-AR comprises a SIL motif. Both the Ser and the Val/Leu in the motif are known to be critical for binding to PDZ domains (43). Accordingly, mutation of the SKV motif sequence of β1-AR (V→A, S→A, and S→D) abrogates binding to the PDZ domain of CNrasGEF (Fig. 1A). CNrasGEF and β1-AR are coexpressed in primary cultured cortical neurons and coronary artery smooth muscle cells (see below). GST-PDZ pull-down experiments with cortical neurons showed that endoge-
FIG. 1. β1-AR, but not β2-AR, binds via its C-terminal SkV motif to CNrasGEF. (A) Pulldown assays with the PDZ domain of CNrasGEF. HEK-293T cells were transfected (tfxn) with either HA-tagged wild-type β1-AR (β1) or mutant β1-AR bearing point mutations in the putative PDZ binding motif, SkV (Val→Ala [VA], Ser→Ala [SA], or Ser→ASP [SD]). Cells were then lysed, and lysates were incubated with either GST alone or a GST fusion protein containing the PDZ domain of CNrasGEF (GST-PDZ). Precipitated proteins were then immunoblotted with anti-HA antibody to detect binding of β1-AR or its mutants. Bottom leftmost lane represents untransfected cell lysate. (B) Same as in panel A, except that β1-AR expressing HEK-293T cells were treated (or not) with a 10 μM concentration of the agonist isoproterenol (Iso) prior to cell lysis and pulldown assays. The phosphatase inhibitor NaF (10 mM) was present during cell treatment and then to become phosphorylated at their C termini by GRK. We thus tested whether a longer treatment (15 min) of the β1-AR expressed in HEK-293T cells with the agonist isoproterenol would alter the ability of this receptor to bind the PDZ domain of CNrasGEF. Our results show that 15 min of isoproterenol treatment in the presence of a phosphatase inhibitor leads to a partial decrease in the binding of β1-AR to the CNrasGEF-PDZ domain (Fig. 1B).

To test whether CNrasGEF and β1-AR can interact in cells (Fig. 3), epitope-tagged version of both proteins (Flag-CNrasGEF and HA-β1-AR) were expressed in HEK-293T cells, which express very small amounts of endogenous CNrasGEF (11; N. Pham and D. Rotin, unpublished data). Under these conditions, a substantial fraction of CNrasGEF colocalizes with the β1-AR at the plasma membrane in HEK-293T cells (Fig. 4A). Moreover, extensive colocalization is also seen in primary cortical neurons (Fig. 4B), and both proteins are also expressed in primary coronary artery smooth muscle cells (Fig. 4C). We then performed coimmunoprecipitation experiments.
As seen in Fig. 3, β1-AR communoprecipitates with CNrasGEF, demonstrating that the two proteins likely associate in cells.

**Physical interaction between β1-AR and CNrasGEF is required for Ras activation upon ligand stimulation.** We next investigated whether CNrasGEF bound to the β1-AR can lead to agonist-induced Ras activation. CNrasGEF and β1-AR were cotransfected into HEK-293T cells, and cells treated with the β adrenergic agonist isoproterenol (10 µM), which stimulates both β1-AR and β2-AR. Such treatment leads to elevation of intracellular cAMP by the β adrenergic receptors (Table 1). Ras activation was then determined by precipitation of active (GTP-bound) Ras with a GST fusion protein of the Ras-binding domain of Raf1 (Raf-RBD) (12). Figure 5A shows that, in response to isoproterenol, β1-AR can stimulate Ras activation only when CNrasGEF is present; this activation is greatly reduced by treatment of the cells with the nonspecific β adrenergic receptor antagonist propranolol (100 µM), indicating that Ras activation is mediated specifically by receptor stimulation. Moreover, the activation is prevented in cells expressing β1-AR bearing a mutation in its SkV motif (β1-AR [V→A]) (Fig. 5A), which cannot bind CNrasGEF (Fig. 1A). Interestingly, isoproterenol treatment of the β2-AR, coexpressed with CNrasGEF, fails to activate Ras (Fig. 5A), a finding in agreement with the inability of β2-AR to bind CNrasGEF (Fig. 1C). The lack of Ras activation with β2-AR or mutant β1-AR (V→A) cannot be attributed to the failure of these receptors to stimulate adenylyl cyclase activity because cAMP production upon stimulation with isoproterenol of β2-AR or mutant β1-AR (V→A) is similar to that of the wild-type β1-AR (Table 1). Both the CDC25 domain and the cNMP-BD of CNrasGEF are required for the stimulation of Ras activation by agonist-induced β1-AR because deletion of these domains (∆CDC25 or ∆cNMP-BD) leads to abrogation of Ras activation by the receptor (Fig. 5B). This suggests that cAMP binding to CNrasGEF and CNrasGEF catalytic activity are necessary for this activation. Collectively, these data demonstrate that the interaction between intact β1-AR and CNrasGEF is required for agonist-dependent stimulation of Ras activation by β1-AR. It is important to note that stimulation of β1-AR in the absence of wild-type CNrasGEF cannot activate Ras in HEK-293T cells (Fig. 5A), indicating that there is no intrinsic cAMP-dependent Ras activation pathway under our experimental conditions. Significantly, our present work demonstrates that Ras activation by agonist-stimulated β1-AR is due to direct activation of CNrasGEF activity via cAMP binding to its cNMP-BD.

**Gsα, but not Gβγ, transduces the β1-AR-CNrasGEF signal.** Signaling downstream of GPCRs can be mediated by various Gα or Gβγ subunits (36). We therefore examined the G-protein subunits that are involved in the β1-AR-dependent Ras activation via CNrasGEF. We first tested whether the Gβγ subunits are involved in this pathway because these subunits had been previously implicated in Ras and ERK activation (36, 39, 40). Overexpression of the transducin α subunit (Gto) (19) or the PH domain of the GPCR kinase (GRK) that physically interact with free βγ (46) can inhibit βγ-mediated signaling, presumably by sequestration of these subunits. As shown in Fig. 6, overexpression of either Gto or the PH domain of Grk does not affect the β1-AR-induced Ras activation via CNrasGEF, suggesting that the Gβγ subunits of G proteins are unlikely to be involved in this pathway.

Because the β1-AR was previously shown to activate adenylyl cyclase via Gsα (29) and because activation of Ras by CNrasGEF requires the generation of cAMP, we tested the effect of the Gsα subunit on the activation of Ras by CNrasGEF. We thus used cholera toxin (CTX) to activate endogenous Gsα. CTX catalyzes the ADP-ribosylation of Gsα, resulting in the inhibition of its intrinsic GTPase activity, which leads to constitutive activation of adenylyl cyclase (51). As shown in Fig. 7A, CTX treatment of HEK-293T cells strongly activated Ras (13-fold) in cells overexpressing Wt-CNrasGEF but not in cells overexpressing the ∆CDC25 or ∆cNMP-BD mutants of CNrasGEF, indicating that stimulation of endogenous Gsα can activate Ras via CNrasGEF. Importantly, activation of endogenous Gsα without CNrasGEF expression could not stimulate

### Table 1. cAMP production in HEK-293T cells expressing β adrenergic receptors and mutant Gsα proteins

<table>
<thead>
<tr>
<th>Cell group</th>
<th>Mean cAMP production ± SEM (pmol/mg of protein)</th>
<th>Without agonist</th>
<th>With agonist</th>
</tr>
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<tbody>
<tr>
<td>Untransfected cells (+ forskolin)</td>
<td>8.59 ± 0.28</td>
<td>47.1 ± 16.9</td>
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<tr>
<td>β1-AR</td>
<td>9.11 ± 3.56</td>
<td>79.2 ± 1.45</td>
<td></td>
</tr>
<tr>
<td>β1-AR (V→A)</td>
<td>7.94 ± 1.48</td>
<td>70.6 ± 3.04</td>
<td></td>
</tr>
<tr>
<td>β2-AR</td>
<td>9.63 ± 2.11</td>
<td>77.9 ± 1.16</td>
<td></td>
</tr>
<tr>
<td>Untransfected cells (+ CTX)</td>
<td>43.3 ± 10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gsα (R201C)</td>
<td>58.6 ± 6.82</td>
<td></td>
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<tr>
<td>Gsα (Q227L)</td>
<td>15.8 ± 2.70</td>
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<tr>
<td>Gsα (R232A/I235A)</td>
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a HEK-293T cells (10⁷) were transfected with vector alone or vector containing the indicated construct. Intracellular cAMP levels were measured by using a cAMP enzyme immunoassay kit (Amersham). HEK-293T cells expressing wild-type β1-AR or β2-AR or mutant β1-AR were stimulated with 10 µM isoproterenol for 15 min. Where indicated, untransfected cells were treated with 50 µM forskolin or 30 ng of CTX/ml. Cells in all treatments were treated with 100 µM IBMX. Data are the means of two independent experiments performed in triplicate.
FIG. 4. Coexpression and colocalization of β1-AR and CNrasGEF. (A) Colocalization of transfected HA-tagged β1-AR (red) and GFP-tagged CNrasGEF (green) in the cell periphery of HEK-293 cells. The β1-AR was detected with anti-HA antibodies. (Ba) Colocalization of endogenous CNrasGEF (green) and β1-AR (red) in primary cultured rat cortical neurons was obtained by double immunostaining with anti-CNrasGEF antibody and anti-β1-AR antibody conjugated with TRITC. (Ba) No β1-AR staining was observed when normal rabbit serum conjugated with TRITC was used as a negative control. (Bb, right panel) Similarly, no CNrasGEF staining was observed when preimmune serum was used as a negative control (Bc, left panel). (C) Endogenous expression of CNrasGEF (green) and β1-AR (red) in coronary artery smooth muscle cells was stained separately.
Ras activation by alternative pathways (e.g., via c-Src) under these experimental conditions (Fig. 7A), demonstrating again that Gs/H9251-dependent Ras activation is mediated directly by CNrasGEF. Constitutively active oncogenic mutations of Gs/H9251 have been described in a subset of endocrine tumors, particularly the R201C and Q227L mutants (30, 35). We thus examined the Gs/H9251-dependent Ras activation via CNrasGEF by these mutant Gs/H9251 proteins in HEK-293T cells. As seen in Fig. 7B, both overexpressed mutant Gs proteins stimulated Ras activation in the presence of Wt-CNrasGEF but not in the presence of its catalytically inactive ΔCDC25 mutant. To verify that the Ras activation (via CNrasGEF) by these two mutants occurs through adenylyl cyclase, we tested the ability of an inactive mutant Gso to activate Ras. Since the cocrystal structure of Gso with the catalytic core of adenylyl cyclase showed that amino acid residues in the switch II region of Gso, as well as those in the α3/β5 loop, directly interact with adenylyl cyclase (21, 44) and, in particular, mutations at the R232 and I235 residues in the switch II region were defective in stimulating adenylyl cyclase in cells (21), we generated the R232A/
I235A mutant of Gsα in the context of the R201C (constitutively active) Gsα mutant. We first verified that this triple mutant exhibits impaired cAMP production (Table 1) and then measured its ability to activate Ras. Figure 7B shows that overexpression of this inactive Gsα triple mutant, along with Wt-CNrasGEF, failed to activate Ras. These data therefore indicate that cAMP produced by activated Gsα is required for Ras activation via CNrasGEF.

**DISCUSSION**

Our previous studies established that CNrasGEF can be activated in response to increased levels of intracellular cAMP (38); however, a physiologically relevant activator had not been identified. Our results here show that the β1-AR is an upstream activator of CNrasGEF. β2-AR, or mutant β1-AR unable to bind to CNrasGEF, cannot activate Ras via CNrasGEF, although these receptors function normally in agonist-induced cAMP production. These data strongly suggest that a physical interaction between β1-AR and CNrasGEF is required for establishing a microenvironment in which CNrasGEF can effectively sense the increasing concentration of cAMP due to receptor stimulation and activate Ras, which is also located at the inner surface of the plasma membrane (Fig. 8). This is the first demonstration of direct physical association between a Ras activator and a GPCR, leading to agonist-induced Ras activation. In this regard, stimulation of β1-AR can directly activate both heterotrimeric G protein, Gsα, and the Ras GEF, CNrasGEF; these proteins therefore act together to activate Ras. Thus, β1-AR-specific Ras activation is distinct from other kinds of G-protein-coupled receptor-mediated ERK activation, including that induced by β2-AR, which primarily employ the indirect Gβγ-dependent c-Src-mediated Grb2-SOS pathway.

Our previous work (38) has demonstrated that strong artificial elevation of cAMP (e.g., by treatment with 8-Br-cAMP or forskolin plus IBMX [3-isobutyl-1-methylxanthine]), coupled with overexpression of CNrasGEF in cells, leads to activation of Ras via CNrasGEF in the absence of β1-AR, as also seen after treatment of cells with CTX or with constitutively active Gsα (Fig. 7). However, under conditions of greatly reduced CNrasGEF expression (when coexpressed with β1-AR or β2-AR; see Fig. 5B) CNrasGEF requires physical interactions with the β1-AR in order to activate Ras. This suggests that, under physiological conditions, the proximity between β1-AR (coupled to Gsα) and CNrasGEF, afforded by their physical interaction, is needed to allow the cAMP generated by agonist stimulation to be sensed by the bound CNrasGEF, which is located in the same microenvironment as the β1-AR. In support of this notion, our results show that activation of β2-AR (which does not bind CNrasGEF), which leads to an elevation of intracellular cAMP similar to that seen with β1-AR, does not cause CNrasGEF-mediated Ras activation. Indeed, a recent report has demonstrated local microdomains with high concentrations of cAMP generated by agonist stimulation of β adrenergic receptors in cardiac myocytes (58), suggesting that...
experiment.

endogenous total Ras. The blots represent one of
depicted expressed proteins in lysates of the transfected cells and of

VOL. 22, 2002

constitutively active Gs

in the context of the R201C mutant. In the presence of Wt-CNrasGEF,

panel) as described above. The R232A/I234A mutation was generated

(R232A/I234A), and cells were processed for Raf-RBD assays (top

tively active Gs

transfected with wild-type or mutant CNrasGEF, along with constitu-

can activate Ras via CNrasGEF.

FIG. 7. β1-AR stimulation of Ras activation by CNrasGEF is trans-
duced via Gso. (A) Endogenous Gso can activate Ras via CNrasGEF. HEK-293T cells were transfected with wild-type or mutant CNrasGEF
(ΔCDC25 or ΔCNMP-BD) and treated with CTX (30 ng) for 90 min.

Cells were then lysed and subjected to Raf-RBD assays. The top panel
shows active Ras-GTP. The lower two panels depict the amount of

and the expression of the CNrasGEF proteins. Blots are

representative of two independent experiments with virtually identical

results. (B) Constitutively active Gso activates Ras via CNrasGEF,

whereas a Gso inhibitor blocks this activation. HEK-293T cells were

transfected with wild-type or mutant CNrasGEF along with constituti-

vively active Gso mutants (R201C or Q227L) or an inactive mutant

(R232A/I234A), and cells were processed for Raf-RBD assays (top

panel) as described above. The R232A/I234A mutation was generated

in the context of the R201C mutant. In the presence of Wt-CNrasGEF,

constitutively active Gso mutants increased Ras activation by 2.5- to

3-fold (n = 5) relative to the Gso mutant alone. The lower panels

depict expressed proteins in lysates of the transfected cells and of

endogenous total Ras. The blots represent one of five independent

experiments.

close proximity to the source of the cAMP generator is impor-
tant for signaling specificity.

The mutant β1-AR bearing the Ser→Asp mutation, which
places a negative charge at the −2 position of the SkV motif,
lost its ability to bind to the PDZ domain of CNrasGEF (Fig.

1A and 2). Moreover, 15 min of stimulation of the receptor,
known to lead to its phosphorylation (presumably on the Ser
of the SkV motif), results in a decrease in binding to the PDZ
domain of CNrasGEF (Fig. 1B). This is consistent with struc-
tural information showing that the hydroxyl side chain of the
serine residue of the SkV/L motif forms a critical hydrogen
bond with a histidine residue in the PDZ domain of PFD-95
(16). In accordance with this, phosphorylation of the inward
rectifier K channel Kir 2.3 on the equivalent serine causes
rapid dissociation of the channel from PSD-95 (6). Moreover,
a recent report has demonstrated that overexpression of intact
GPCR kinase 5 (GRK5) decreases β1-AR association with the
PDZ domain of PFD-95 (26). This reduction of β1-AR-
PFD-95 interaction is mimicked by receptor stimulation with
an agonist, but a kinase-inactive GRK5 mutant has no effect on
PFD-95 binding to β1-AR. Thus, it is possible that phos-
phorylation of the serine in the SkV motif of the β1-AR, commonly
seen after the stimulation of GPCRs (29), leads to dissociation
of the CNrasGEF-PDZ domain from the receptor, thus termi-
nating the signal for Ras activation (Fig. 8).

Although the βγ subunits of G proteins have been known to
play an important role in GPCR-induced ERK activation,
overexpression of βγ subunits fails to induce cell proliferation
(8, 9). In contrast to Gαγ, mutations or aberrant expression of
GPCRs or their associated Gα proteins has been linked to
several cancers (36). Accordingly, activating mutations in dif-
ferent Gα subunits cause cellular transformation in cultured
fibroblasts (14) via various mechanisms. In the case of consti-
tutively active mutant Gia, cell proliferation is caused by up-
regulation of Ras-independent ERK activation, which is a re-
sult of diminished cAMP-PKA inhibition of Raf-1 activation
(50, 54) in tumors, particularly in those of endocrine origin
(30, 35, 49). In these tumors, the activating mutations in both Gso
and the thyroid-stimulating hormone receptor (a GPCR) re-
sult in the constitutive activation of adenyl cyclase (48). Fur-
thermore, increases in cAMP levels, β1-AR function (17, 42),
and Ras activity (2, 45) were independently reported in cardiac
hypertrophy, but the underlying mechanisms are unknown.

Thus, although speculative, it is possible that CNrasGEF is
involved in pathological conditions where β1-AR, cAMP, or
Gso are implicated.

cAMP has been traditionally known to inhibit cell growth via
PKA-dependent phosphorylation of Raf-1 and inhibition of the
Erk pathway in various cell types (7, 55), except for neu-
rons and endocrine cells, where it is stimulatory. For example,
in thyroid cells and pituitary growth hormone-secreting cells,
marked elevation of intracellular cAMP due to activating mu-
tations in Gso results in cellular transformation, which was
proposed to arise from persistent activation of PKA (30, 35).
However, it was recently shown that thyroid-stimulated cell
proliferation is not completely blocked by a PKA inhibitor and
that this additional cAMP-dependent, PKA-independent cell
proliferation could be due to activation Ras (5, 47). In addition
to activating PKA, cAMP can also stimulate the Rap1 ex-
change factors Epac or Rap-GEF/II (13, 28), leading to Rap1,
B-Raf, and Erk activation (3). However, in the neurocrest-derived B16 melanocytes, cAMP-mediated activation of Erk is independent of Epac/Rap1 (or PKA) stimulation and instead involves Ras activation (4). Thus, there is clear evidence for the existence of a cAMP-mediated, a PKA-independent, and a Rap1-independent pathway(s) for Ras activation in cells, where CNrasGEF may be involved.

The scaffold protein MAGI-2 (S-SCAM) has been demonstrated to bind CNrasGEF (37) and, independently, to bind β1-AR (57). It is unlikely, however, that the β1-AR-stimulated activation of CNrasGEF reported here is mediated via MAGI-2 because its binding to the β1-AR would preclude CNrasGEF binding due to competition for the same binding site (37) and because we demonstrated direct binding between β1-AR and CNrasGEF (Fig. 2). Moreover, MAGI-2 is not expressed in the heart (37), a key organ regulated by the β1-AR, which expresses both β1-AR and CNrasGEF.

In summary, unlike other GPCRs, the β1-AR activates Ras with a distinct mechanism, requiring direct physical interaction with the Ras activator CNrasGEF and an active role of Gs instead of Gβγ. This β1-AR-stimulated Ras activation by CNrasGEF is an important alternative pathway for activating Ras independent of PKA and is likely physiologically relevant in both the neuronal and cardiovascular systems. In the latter, the activation of CNrasGEF and Ras specifically by β1-AR and not β2-AR may have pharmacological ramifications for the management of cardiovascular disease states, where a selective inhibition of β1-AR alone would eliminate the undesirable side effects of β blockade on β2-AR expressing organs, such as pulmonary airways.

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Binding of G protein beta gamma-subunits to pleckstrin homology domains.