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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 Gsα 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 Gsα-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

G-protein-coupled receptors (GPCRs) have been classically known to transduce extracellular signals intracellularly by utilizing heterotrimeric G protein complexes comprised of α, β, and γ subunits. Agonist binding promotes a conformational change in GPCRs, leading to the release of βγ and guanine nucleotide exchange on the α subunit that loads it with GTP, which in turn can stimulate a number of intracellular second messengers, such as adenylyl cyclase and phospholipase C. However, this model alone cannot adequately explain the full range of effects of GPCRs, especially the stimulation of tyrosine phosphorylation of cellular proteins, the activation of mitogen-activated protein kinase cascades, cell growth, and proliferation (15, 34, 53). This GPCR-mediated, Ras-dependent mitogen-activated protein activation, especially the activation of extracellular-signal-regulated kinase (ERK), has been explained by indirect mechanisms through either Gβγ or Gqα and cross talk to tyrosine kinases. The free Gβγ subunits generated upon ligand binding to GPCRs have been known to recruit c-Src/phosphatidylinositol 3-kinase (PI3K), which phosphorylates SHC, leading to Grb2-Sos1 recruitment and Ras activation by Sos1 (36). Either overexpression of a truncated inhibitory mutant of c-Src or treatment with the PI3K inhibitor wortmannin, effectively blocked the Gβγ-induced Ras activation (10, 23), implicating c-Src and PI3K in this pathway. In the case of the β2 adrenergic receptor (β2-AR), however, β-arrestin has been found to play a role to recruitment and activation of c-Src, and β-arrestin mutants impaired in either c-Src binding or internalization of GPCR cannot mediate β2-AR-induced ERK activation (33), suggesting that this internalization mediator is important for ERK activation. Here, as in prior studies, Gβγ has been shown to have a central role in Src-dependent ERK activation because Gβγ recruits GRK2 to the plasma membrane, with the consequent phosphorylation of the receptor by GRK2 and the ensuing recruitment of β-arrestin to the phosphorylated (activated) GPCR.

Another indirect way to activate Ras after GPCR stimulation is via the Gqα-PLCβ pathway and activation of Pyk2 (31), which is independent of Gβγ (10, 24). Pyk2 is activated by increased intracellular Ca^{2+} and then phosphorylates SHC. The resulting SHC-Grb2-Sos1 complex stimulates Ras activation (31). Thus, all of these mechanisms for GPCR-induced Ras/ERK activation are indirect, through the released Gβγ or Gqα and cross talk to tyrosine kinases such as c-Src.

The activation of ERK by cyclic AMP (cAMP) has been reported in a limited number of cell types, including PC12 (20, 52) and B16 melanoma cells (4, 18). In PC12 cell, increased intracellular cAMP activates protein kinase A (PKA), which subsequently phosphorylates Rap-1, which in turn activates B-Raf, leading to Erk activation (52). As in PC12 cells, cAMP also induces ERK activation in B16 melanocytes. However, this activation is dependent on Ras and independent of PKA, Epac/Rap-1, or Sos (4). This cAMP-dependent but PKA-independent Ras activation has been also reported in neuronal cells and cardiac cells (1, 41), but the mechanisms involved are unknown. Thus, these studies suggest the existence of a novel cAMP-dependent Ras activation pathway in these cells.

We have previously identified the cyclic nucleotide Ras GEF

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(CNrasGEF) in a screen for Nedd4-interacting proteins (38). CNrasGEF (also known as PDZ-GEF1, nRap-GEP, or RA-GEF [11, 32, 37]) is a 180-kDa protein containing a CDC25 (guanine nucleotide exchange factor [GEF]) domain, a PDZ domain, a cyclic nucleotide binding domain (cNMP-BD), and other domains and motifs (38). It can activate Rap1 independently of cAMP (11, 32, 37, 38) and, importantly, it can directly bind cAMP and activate Ras in response to elevated intracellular cAMP in a PKA-independent manner (38). However, the physiological upstream receptor(s) and/or activator(s) of CNrasGEF was not known. Here we show that β1-AR, but not β2-AR, binds directly to the PDZ domain of CNrasGEF, leading to agonist-induced Ras activation by the cAMP generated from activated Gsα. Moreover, direct binding of CNrasGEF to the β1-AR is necessary for Ras activation by CNrasGEF. Thus, agonist signal can be directly converted into Ras activation through the β1-AR-associated CNrasGEF. These findings provide the first demonstration of a direct binding of a Ras activator to a GPCR and the ensuing agonist-mediated activation of Ras via this Ras GEF.

MATERIALS AND METHODS

Constructs and antibodies. Hemagglutinin (HA)-β1-AR was inserted into pCDNA3. The mutant β1-AR with changes in the carboxyl-terminal SKV sequence were generated by PCR, verified by sequencing, and subcloned into pCDNA3. The His-tagged β1-AR carboxyl tail (His-β1-Ct, corresponding to amino acids 425 to 477) and mutant His-β1-Ct were generated by PCR and subcloned into pQE40 (Qiagen). Bacterial His-β1-Ct proteins were purified according to the manufacturer's instructions. The pleckstrin homology (PH) domain (corresponding to amino acids 587 to 681) of GRK2 was amplified by PCR, tagged with MRGS-His_6, and subcloned into pCDNA3. Wild-type G-protein α subunit (Gsα) was tagged with EE epitope (EYMPME) and inserted into pCDNA3. The different Gsα mutants were generated by PCR, verified by sequencing, and inserted into pCDNA3. CNrasGEF (Wt, ΔCDC25, and ΔcNMP-BD) for mammalian expression and the glutathione *S*-transferase (GST)–PDZ constructs were as previously described (38). Monoclonal anti-His antibody was from Qiagen, and monoclonal anti-EE antibody was from BAbCO. Polyclonal antibody against β1-AR was from Santa Cruz. Anti Flag antibodies from Sigma and anti HA antibodies from BAbCO. Polyclonal antibodies against CNrasGEF were characterized previously (38). TRITC (tetramethyl rhodamine isothiocyanate) was purchased from Pierce.

Cell culture and transfections. HEK-293T cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 100 U of penicillin, and 100 μg of streptomycin per ml. Primary cultures from rat cortex were prepared from embryonic day 18 to day 20 fetuses, as described previously (56), and primary coronary artery smooth muscle cells were prepared from neonatal pigs (2 to 4 days old) according to a previously described method (25). HEK-293T cells and neurons were transfected by calcium phosphate precipitation methods or by using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol.

Pulldown assay, in vitro binding experiments, and coimmunoprecipitation. HEK-293T cells expressing either wild-type or mutant β1-AR, as well as cultured primary cortical neurons, were lysed with 1 ml of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 10 mg of leupeptin/ml, 10 mg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation at 14,000 rpm for 15 to 20 min. The cleared supernatants were used for pulldown and coimmunoprecipitation experiments. For pulldown experiments, cell lysates (500 μg) were incubated with 50 μg of GST or GST-CNrasGEF-PDZ (GST-PDZ) protein on glutathione-Sepharose beads for 2 h at 4°C. Beads were washed twice with 1 ml of high-salt HNTG (20 mM HEPES [pH 7.5], 500 mM NaCl, 10% glycerol, 0.1% Triton X-100) and twice with HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Bound proteins were eluted from the beads with 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membrane. Bound β1-AR was identified with anti-HA antibody (1:10,000) or β1-AR antibody (1:5,000), followed by treatment with secondary antibodies and enhanced chemiluminescence detection (Amersham). For the in vitro binding experiment, His-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 coimmunoprecipitations, HEK-293T cell lysates expressing either untransfected or transfected HA-β1-AR and Flag-CNrasGEF (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).

Immunofluorescent 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 cultures was stained with anti-CNrasGEF antibody (1:200) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (1:300). 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 (1:200), and then stained with anti-β1-AR antibody, which was conjugated with TRITC (1:50). 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 $NaVO_4$, 10 mM $MgCl_2$, 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 (10^6 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 SlL 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 pulldown experiments with cortical neurons showed that endoge-

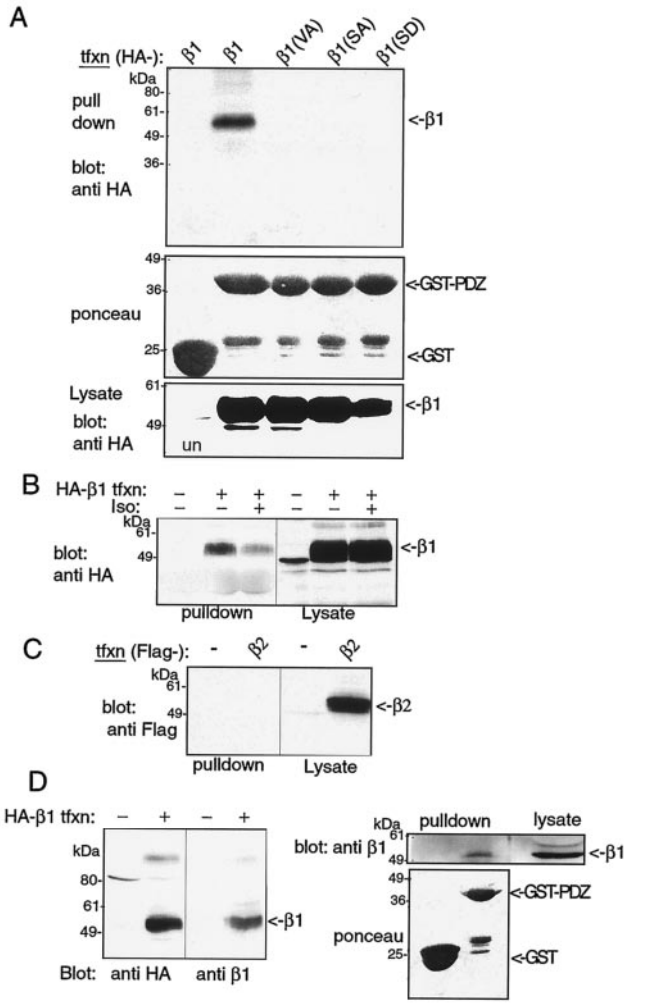

FIG. 1. β1-AR, but not β2-AR, binds via its C-terminal SkV motif to CNrasGEF.(A) Pulldown assays with the PDZ domain of CNrasGEF. HEK-293T cells were transfected (tfxn) with either HA-tagged wild-type β1-AR (β1) or mutant β1-AR bearing point mutations in the putative PDZ binding motif, SkV (Val→Ala [VA[, 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 lysis and the pulldown assays. The results shown represent six of seven independent experiments. (C) HEK-293T cells transfected with Flag-tagged β2-AR were lysed as in panel A above, and a pulldown assay performed with GST-PDZ, followed by anti-Flag immunoblotting to detect the β2-AR. No precipitation of the β2-AR was detected despite strong expression of the protein. (D) In the right panel are shown pulldown assay results with GST-PDZ of β1-AR expressed endogenously in primary cultured cortical neurons. Precipitated β1-AR was immunoblotted with anti β1-AR. Ponceau S-stained nitrocellulose shows the total GST or GST-PDZ proteins used in the pulldown experiments in panels A to C. Ten percent of the lysates used for the pulldown experiments in panels A to C are also depicted. The left panel shows parallel immunoblots with anti-β1-AR antibodies and anti-HA antibodies of lysates expressing HA-β1-AR to demonstrate the cleanliness of the anti-β1-AR antibodies used in our studies.

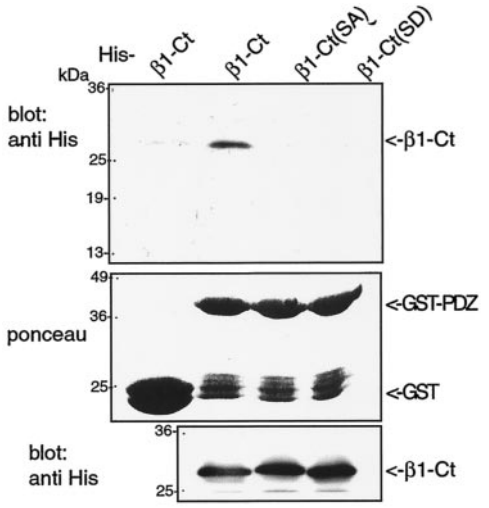

FIG. 2. Direct binding of the PDZ domain of CNrasGEF to the C terminus of β1-AR. Equal amounts (20 μg) of purified His-tagged fusion proteins corresponding to the C terminus of β1-AR (β1-Ct) or the C terminus bearing mutations in the Ser of the SkV motif (β1-Ct [SA] or β1-Ct [SD]) were incubated with GST or GST-PDZ. β1-Ct binding to the GST-PDZ was detected by immunoblotting with anti-His antibodies (upper panel). The lower two panels depict the amounts of the GST/GST-PDZ and His-β1-Ct used for the experiment.

nous β1-AR can also bind to the PDZ domain of CNrasGEF (Fig. 1D, right panel). Upon agonist stimulation, β adrenergic receptors are known to rapidly activate adenylate cyclase (within less than a min), leading to the generation of cAMP, 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 determine whether the interaction between the β1-AR and CNrasGEF is direct, we performed in vitro binding assays with a purified His-tagged C-terminal tail of β1-AR (His-β1-Ct) and purified GST-PDZ domain of CNrasGEF. Our results show that the C terminus of β1-AR can directly bind the PDZ domain of CNrasGEF (Fig. 2). This binding is prevented, however, in β1-Ct bearing an S→A or an S→D mutation in the SkV motif (Fig. 2). These results demonstrate a direct interaction between the PDZ domain of CNrasGEF and the SkV motif of the β1-AR.

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.

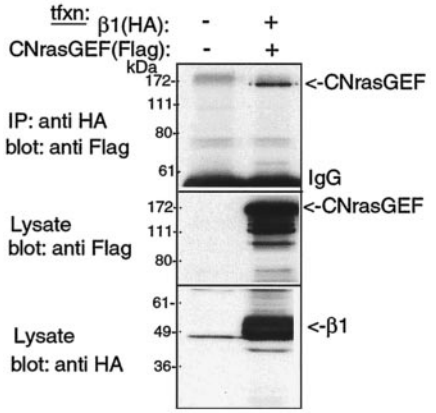

FIG. 3. Coimmunoprecipitation of CNrasGEF and β1-AR coexpressed in HEK-293T cells. Cells expressing HA-tagged β1-AR and Flag-tagged CNrasGEF were lysed, β1-AR immunoprecipitated with anti-HA antibodies and coprecipitated proteins immunoblotted with anti-Flag antibodies. The lower panels depict the expression of the transfected proteins in the cell lysates. The percentage of coimmunoprecipitated proteins relative to total proteins expressed is low.

As seen in Fig. 3, β1-AR coimmunoprecipitates 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 (ΔCDC25 or ΔcNMP-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.

TABLE 1. cAMP production in HEK-293T cells expressing β adrenergic receptors and mutant Gsα proteins[a]

Cell group	Mean cAMP production ± SEM (pmol/mg of protein)	
	Without agonist	With agonist
Untransfected cells (+forskolin)	8.59 ± 0.28	47.1 ± 16.9
β1-AR	9.11 ± 3.56	79.2 ± 1.45
β1-AR (V→A)	7.94 ± 1.48	70.6 ± 3.04
β2-AR	9.63 ± 2.11	77.9 ± 1.16
Untransfected cells (+CTX)		69
Gsα (R201C)	43.3 ± 10.0	
Gsα (Q227L)	58.6 ± 6.82	
Gsα (R232A/I235A)	15.8 ± 2.70	

[a] HEK-293T cells (10^6) 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.

Gsα, but not Gβγ, transduces the β1-AR-CNrasGEF signal. Signaling downstream of GPCRs can be mediated by various Gα or βγ 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 βγ 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 (Gtα) (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 Gtα or the PH domain of GRK does not affect the β1-AR-induced Ras activation via CNrasGEF, suggesting that the βγ 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 ΔCDC25 or ΔCNMP-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

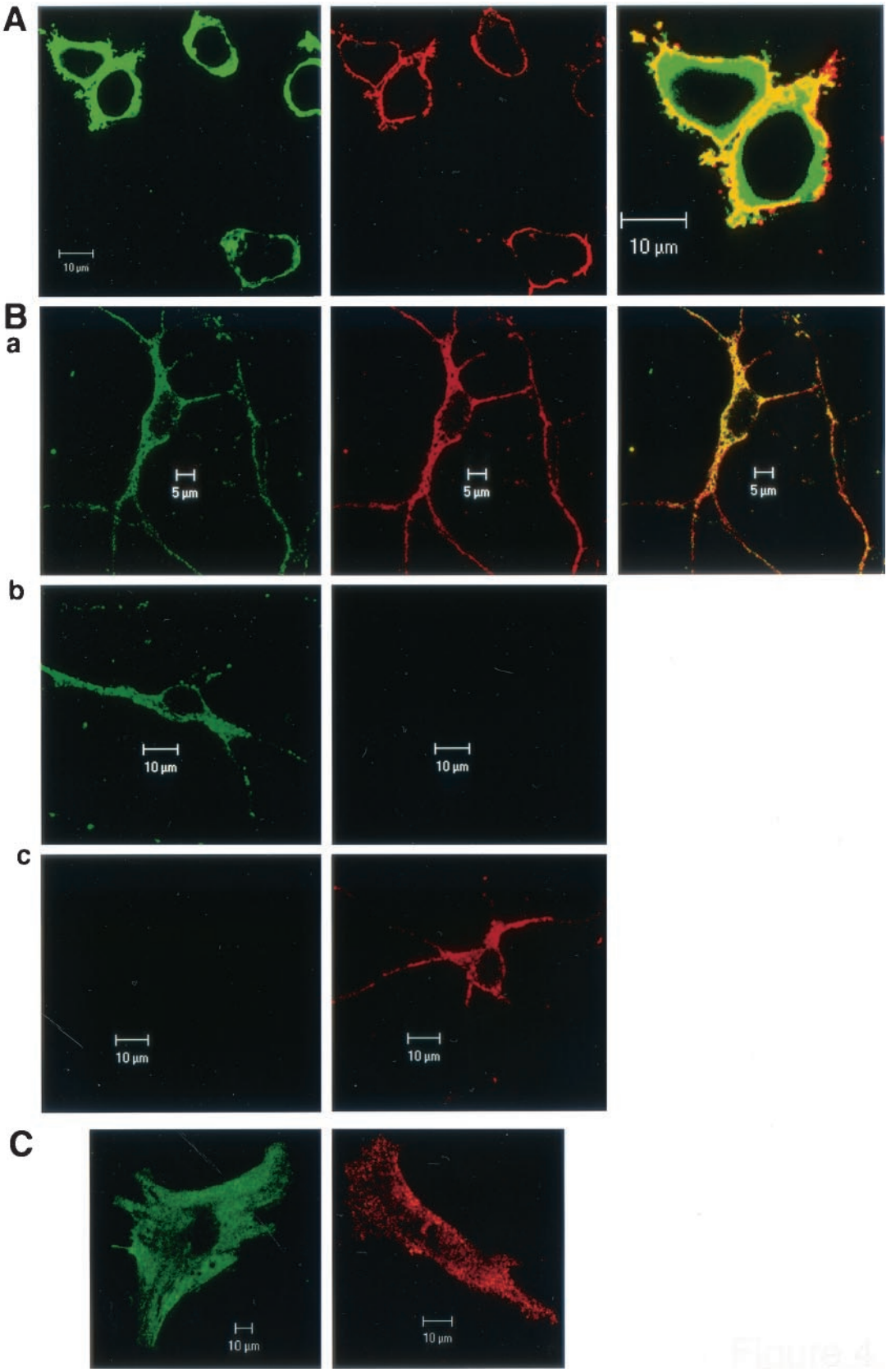

FIG. 4. Coexpression and colocalization of β1-AR and CNrasGEF. (A) Colocalization of transfected HA-tagged β1-AR (red) and GFP-tagged CNrasGEF (green) in the cell periphery of HEK-293 cells. The β1-AR was detected with anti-HA antibodies. (Ba) Colocalization of endogenous CNrasGEF (green) and β1-AR (red) 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.

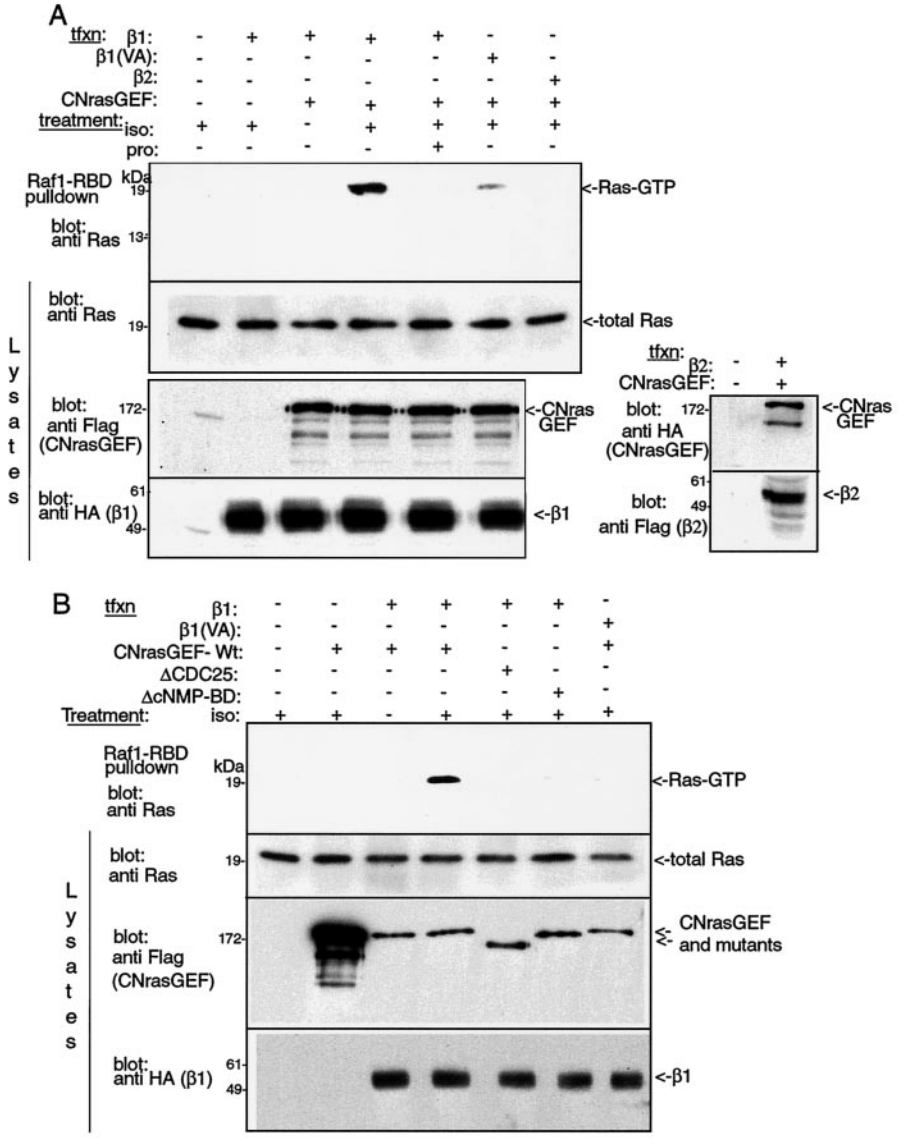

FIG. 5. Stimulation of β1-AR leads to Ras activation via CNrasGEF in cells. (A) Wild-type β1-AR, but not mutant β1-AR that cannot bind CNrasGEF or β2-AR, activates Ras in the presence of CNrasGEF. HEK-293T cells were transfected with either β1-AR, β1-AR (VA), or β2-AR, along with CNrasGEF, serum starved overnight, and then treated with 10 μM isoproterenol (iso) for 15 min with (+) or without (−) 100 μM propranolol (pro). Cells were then lysed and incubated with immobilized GST-Raf1-RBD (Raf-RBD) to precipitate active (GTP-bound) Ras (11). Ras-GTP was detected with anti-Ras antibodies as shown in the top panel. The lower three panels show the amounts of total Ras (endogenous) and transfected β-adrenergic receptors and CNrasGEF. In the presence of Wt-CNrasGEF, isoproterenol increased Ras activation by 10.9-fold ($n = 9$). (B) β1-AR-mediated stimulation of Ras requires the presence of intact CNrasGEF. The experiment was performed as in panel A, but mutant CNrasGEF lacking either the catalytic CDC25 domain (ΔCDC25) or the cyclic nucleotide binding (ΔCNMP-BD) domain was expressed together with β1-AR. The data shown are representative of four independent experiments.

Ras activation by alternative pathways (e.g., via c-Src) under these experimental conditions (Fig. 7A), demonstrating again that Gsα-dependent Ras activation is mediated directly by CNrasGEF. Constitutively active oncogenic mutations of Gsα have been described in a subset of endocrine tumors, particularly the R201C and Q227L mutants (30, 35). We thus examined the Gsα-dependent Ras activation via CNrasGEF by these mutant Gsα proteins in HEK-293T cells. As seen in Fig. 7B, both overexpressed mutant Gsα 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 Gsα to activate Ras. Since the cocrystal structure of Gsα with the catalytic core of adenylyl cyclase showed that amino acid residues in the switch II region of Gsα, 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/

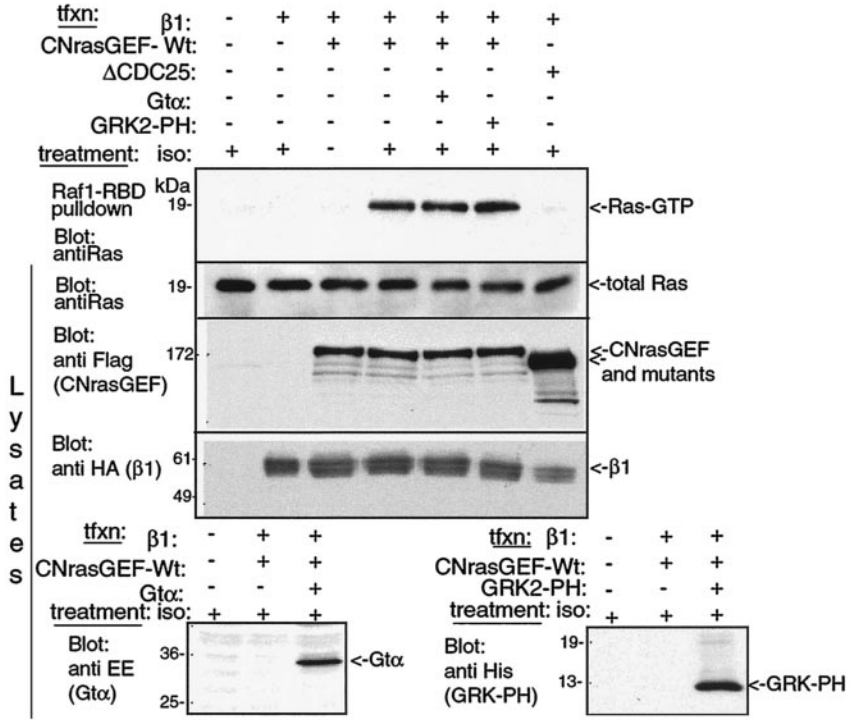

FIG. 6. β1-AR stimulation of Ras activation by CNrasGEF is not mediated via Gβγ. (A) HEK-293T cells were transfected with either the transducin α-subunit (EE-tagged Gtα) or the PH domain of GRK2 (His-tagged GRK-PH), along with HA-tagged β1-AR and Flag-tagged CNrasGEF. Cells were treated with 10 μM isoproterenol for 15 min and lysed, and the lysates were then subjected to a Raf-RBD assay to detect active Ras (Ras-GTP, top panel). Expression levels of total (endogenous) Ras and all transfected constructs in cell lysates are depicted in the lower panels.

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

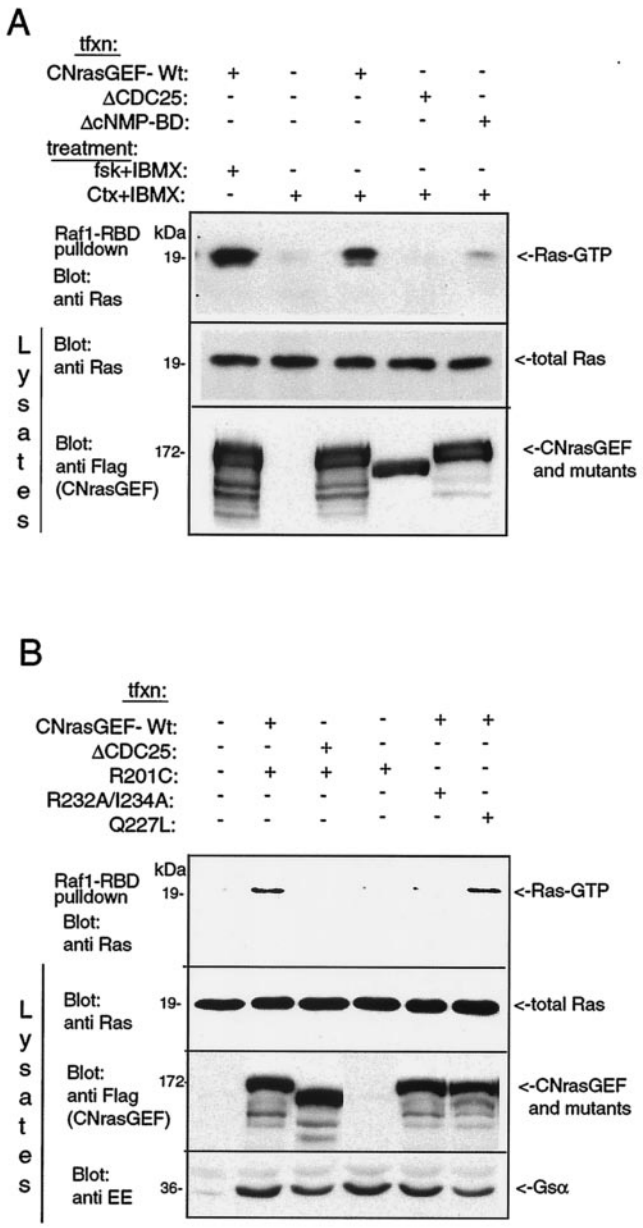

FIG. 7. β1-AR stimulation of Ras activation by CNrasGEF is transduced via Gsα. (A) Endogenous Gsα can activate Ras via CNrasGEF. HEK-293T cells were transfected with wild-type or mutant CNrasGEF (ΔCDC25 or ΔCNMP-BD) and treated with CTX (30 ng) for 90 min. Cells were then lysed and subjected to Raf-RBD assays. The top panel shows active Ras-GTP. The lower two panels depict the amount of total Ras and the expression of the CNrasGEF proteins. Blots are representative of two independent experiments with virtually identical results. (B) Constitutively active Gsα activates Ras via CNrasGEF, whereas a Gsα inhibitor blocks this activation. HEK-293T cells were transfected with wild-type or mutant CNrasGEF, along with constitutively active Gsα 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 Gsα mutants increased Ras activation by 2.5- to 3-fold ($n = 5$) relative to the Gsα 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 important 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 structural information showing that the hydroxyl side chain of the serine residue of the SxV/L motif forms a critical hydrogen bond with a histidine residue in the PDZ domain of PSD-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 PSD-95 (26). This reduction of β1-AR–PSD-95 interaction is mimicked by receptor stimulation with an agonist, but a kinase-inactive GRK5 mutant has no effect on PSD-95 binding to β1-AR. Thus, it is possible that phosphorylation of the serine in the SxV 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 terminating 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 Gβγ, mutations or aberrant expression of GPCRs or their associated Gα proteins has been linked to several cancers (36). Accordingly, activating mutations in different Gα subunits cause cellular transformation in cultured fibroblasts (14) via various mechanisms. In the case of constitutively active mutant Giα, cell proliferation is caused by upregulation of Ras-independent ERK activation, which is a result 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 Gsα and the thyroid-stimulating hormone receptor (a GPCR) result in the constitutive activation of adenylyl cyclase (48). Furthermore, 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 Gsα 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 neurons 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 mutations in Gsα 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 exchange factors Epac or Rap-GEFI/II (13, 28), leading to Rap1,

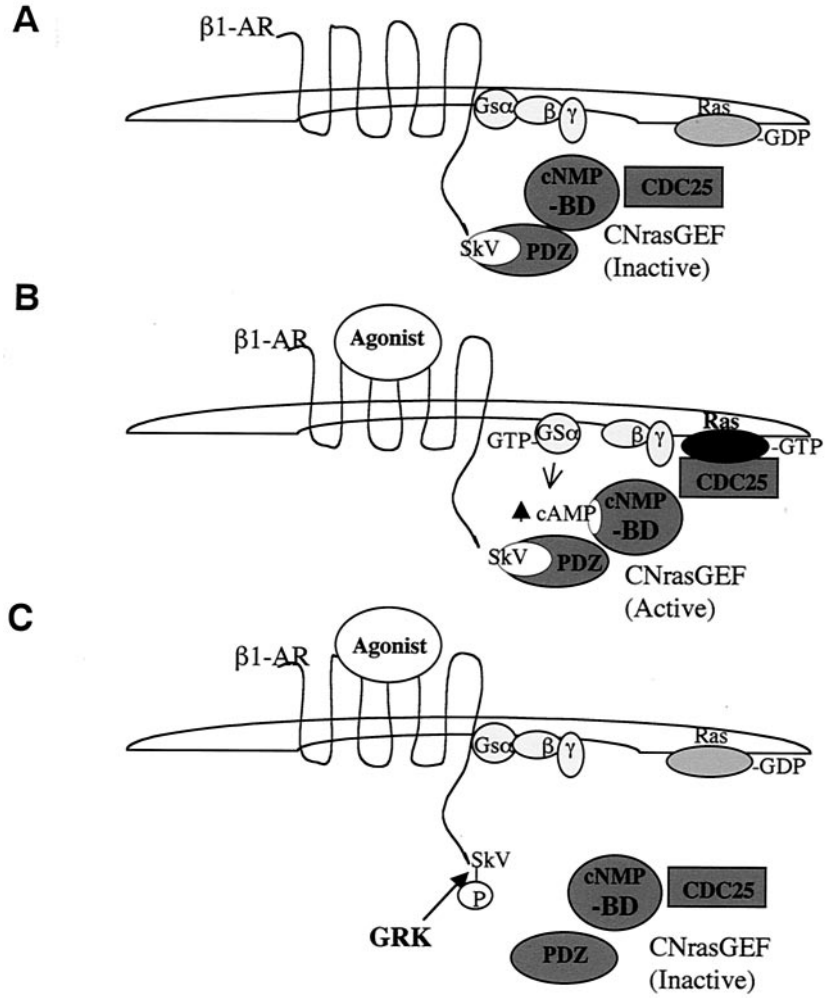

FIG. 8. Model for β1-AR–mediated Ras activation via CNrasGEF. (A) In the basal state, the β1-AR is associated with the PDZ domain of CNrasGEF via its PDZ binding motif (SkV motif). (B) Agonist binding to the β1-AR results in the dissociation of heterotrimeric G proteins and the activation of Gsα (Gsα-GTP), which in turn activates adenylyl cyclase. Consequently, elevated intracellular cAMP binds to the cNMP-BD of CNrasGEF, activating the catalytic activity of CNrasGEF and leading to Ras activation. (C) After β1-AR stimulation (i.e., after the elevation of cAMP), the serine residue of the SkV motif in the β1-AR carboxyl tail is phosphorylated by GRK2 or GRK5 (GRK), leading to subsequent dissociation of the PDZ domain of CNrasGEF from the β1-AR. For simplicity, only the relevant domains of CNrasGEF (cNMP-BD, PDZ, and CDC25, all in dark gray) are indicated.

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 Gsα 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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