RACK1 Regulates Specific Functions of G\(\beta\gamma\)*

Received for publication, December 15, 2003, and in revised form, January 22, 2004
Published, JBC Papers in Press, February 12, 2004, DOI 10.1074/jbc.M313727200

Songhai Chen‡§, Edward J. Delli‡, Fang Lin‡, Jiqing Sai¶‖, and Heidi E. Hamm‡§

From the Departments of ‡Pharmacology and ¶Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee 37232-6600.

We showed previously that G\(\beta\gamma\) interacts with Receptor for Activated C Kinase 1 (RACK1), a protein that not only binds activated protein kinase C (PKC) but also serves as an adaptor/scaffold for many signaling pathways. Here we report that RACK1 does not interact with Ga subunits or heterotrimeric G proteins but binds free G\(\beta\gamma\) subunits released from activated heterotrimeric G proteins following the activation of their cognate receptors in vivo. The association with G\(\beta\gamma\) promotes the translocation of RACK1 from the cytosol to the membrane. Moreover, binding of RACK1 to G\(\beta\gamma\) results in inhibition of G\(\beta\gamma\)-mediated activation of phospholipase C \(\beta\)2 and adenylyl cyclase II. However, RACK1 has no effect on other functions of G\(\beta\gamma\), such as activation of the mitogen-activated protein kinase signaling pathway or chemotaxis of HEK293 cells via the chemokine receptor CXCR2. Similarly, RACK1 does not affect signal transduction through the Go subunits of Gi, Go or Gq. Collectively, these findings suggest a role of RACK1 in regulating specific functions of G\(\beta\gamma\).

Heterotrimeric G proteins transduce extracellular signals from a large family of G protein-coupled receptors and mediate intracellular responses critical for many cellular processes, such as vision, taste, metabolism, and neuronal and cardiovascular functions (1). G proteins consist of three subunits, \(\alpha\), \(\beta\), and \(\gamma\). The signaling function of G proteins was once attributed totally to the Go subunit. The G\(\beta\gamma\) subunit was considered merely a membrane anchor and a negative regulator of the Ga. However, it is now clear that it itself plays a prominent role in signal transduction. G\(\beta\gamma\) has a long list of effector and interacting proteins and has been shown to play a dominant role in certain cellular functions. For example, in yeast, G\(\beta\gamma\) is the principal transducer of the mating signal for cell cycle arrest and differentiation (2). Chemotactic responses of leukocytes (3), 4 and Dictyostelium discoideum amoeba (5, 6) are mediated through G\(\beta\gamma\). Recently, G\(\beta\gamma\) has also been implicated in smooth muscle cell proliferation and arterial restenosis (7).

The diversity of G\(\beta\gamma\) target proteins raises the question of how the specificity and efficiency of G\(\beta\gamma\) signaling are regulated. Several G\(\beta\gamma\)-interacting proteins have been shown to regulate its function. For example, phosducin and phosducin-like proteins (PhLPs)\(^1\) bind G\(\beta\gamma\) with high affinities and are regarded as scavengers of G\(\beta\gamma\) (8). Binding of these proteins to G\(\beta\gamma\) limits the amount of G\(\beta\gamma\) available to interact with Ga and to form functional G protein heterotrimers, resulting in inhibition of signal transmission from receptors to G proteins (9, 10). Moreover, they block the activation of effectors by G\(\beta\gamma\), because their binding sites on G\(\beta\gamma\) overlap with the contact residues for G\(\beta\gamma\) effectors (11, 12). Some G\(\beta\gamma\) effectors, such as G protein-coupled receptor kinases (GRK) 2 and 3, could also exert regulatory roles in G\(\beta\gamma\) signaling, because binding of these proteins impede the access of other effectors to G\(\beta\gamma\) (13). Notably, these G\(\beta\gamma\)-interacting proteins appear to affect various functions of G\(\beta\gamma\). However, whether they could modulate the signaling specificity of G\(\beta\gamma\) remains unknown.

Recently, through the use of a yeast two-hybrid screen, glutathione S-transferase (GST)-pull-down and co-immunoprecipitation studies, we identified a new G\(\beta\gamma\)-binding protein, RACK1 (14). RACK1 is a WD40 repeat protein that is proposed to form a seven-bladed \(\beta\) propeller, similar to that formed by G\(\beta\gamma\) (15). Although originally identified as a receptor for binding and translocating activated PKC to particular cell fractions, subsequent studies indicate that it can also act as an adaptor/scaffold protein to recruit or assemble many other signaling proteins into membrane-associated complexes (15).

We showed previously that RACK1 interacts not only with the G\(\beta_i\)\(\gamma_i\) subunit but also heterotrimeric Ga\(\beta_i\)\(\gamma_i\) (14). Moreover, G\(\beta_i\)\(\gamma_i\) competes with several RACK1-interacting proteins such as PKC\(\beta\)II and dynamin-1 for binding to RACK1 (14). However, the functional significance for the interaction between RACK1 and G\(\beta\gamma\) on G protein signaling remains unknown.

In this study, we provide evidence that RACK1 interacts only with free G\(\beta\gamma\) subunits released from activated heterotrimeric G proteins in cells. Moreover, we show that the binding of G\(\beta\gamma\) to RACK1 results in the translocation of RACK1 from the cytosol to the plasma membrane, and the association of RACK1 with G\(\beta\gamma\) specifically inhibits G\(\beta\gamma\) subunit-mediated signal transduction. Particularly, only a subset of G\(\beta\gamma\) functions is affected by RACK1. To our knowledge, this is the first protein identified to be able to regulate specific functions of G\(\beta\gamma\).

EXPERIMENTAL PROCEDURES

Materials—Cyclic AMP assay kits were from R&D systems, myo-[\(^3\)H]inositol (80 Ci/mmol) and phosphatidylinositol-4,5-biphosphate (5.5

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by the National Institutes of Health Grant CA34590-20.

‡ To whom correspondence may be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, Rm. 442, Robinson Research Bldg., Nashville, TN 37232-6600. Tel.: 615-343-3533; Fax: 615-343-1084; E-mail: Songhai.Chen@Vanderbilt.edu (S. C.) or Heidi.Hamm@Vanderbilt.edu (H. E. H.).

§ The abbreviations used are: PhLP, phosducin-like protein; GRK, G protein-coupled receptor kinase; AC, adenylyl cyclase; DSP, dithio-bis(succinimidylpropionate); GST, glutathione S-transferase; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; PI, phosphatidylinositol; PLC, phospholipase C; RACK1, receptor for activated C kinase; PKC, protein kinase C; AR, adrenergic receptor; CMV, cytomegalovirus; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein; GTP\(\gamma\)S, guanosine 5’-3-O-(thio)-triphosphate; IL-8, interleukin-8.

---

1 The abbreviations used are: PhLP, phosducin-like protein; GRK, G protein-coupled receptor kinase; AC, adenylyl cyclase; DSP, dithio-bis(succinimidylpropionate); GST, glutathione S-transferase; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; PI, phosphatidylinositol; PLC, phospholipase C; RACK1, receptor for activated C kinase; PKC, protein kinase C; AR, adrenergic receptor; CMV, cytomegalovirus; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein; GTP\(\gamma\)S, guanosine 5’-3-O-(thio)-triphosphate; IL-8, interleukin-8.
Phosphatididylinositol Hydrolisis in Intact Cells—Phosphatidylinositol (PI) hydrolysis in intact, transfected COS-7 cells was determined as described previously (21, 22). To determine the effect of pertussis toxin on PI hydrolysis, cells were preincubated with pertussis toxin (0.5 μg/ml) overnight before PI measurement.

Measurement of PLCβ2 Activity—The Gβγ-mediated PLCβ2 activation was determined essentially as described (17), except that purified Gβγ2 from S9 cells instead of S9 cell lysates was used in the assay. To determine the effect of RACK1, MBP-RACK1 was preincubated with Gβγ for 30 min before the addition of PLCβ2 and lipid vesicles.

AC Assays—The Gβγ-mediated ACII activation was determined using cell membranes prepared from ACII baculovirus-infected S9 cells as described, except that GTPγS-bound wild-type Gαi instead of the constitutively active mutant Gαi (Q227L) was used (17). To determine the effect of RACK1, MBP-RACK1 was preincubated with Gβγ for 30 min before addition into the reaction. The reaction was terminated by the addition of 0.1 M HCl. CAMP levels were determined using the cAMP immunoassay system (R & D Systems) for nonacetylated cAMP according to the manufacturer’s instructions.

MAPK Activity—HEK293 cells transiently transfected with the αoA, AR, GST, GST-RACK1, or Gαo, cDNAs as described above. Chemotaxis assays were performed in 96-well Boyden chambers (23).

Measurement of Cyclic AMP in Intact Cells—HEK293 cells stably transfected with CXC2 chemokine receptor were used in this assay. Forty-eight hours after transfection with GST, GST-RACK1, or RACK1-V5-His, HEK293 cells were incubated with 0.1% bovine serum albumin in serum-free medium overnight. To determine the endogenous β2-AR-mediated CAMP accumulation, cells were preincubated with 250 μM 3-isobutyl-1-methylxanthine and 20 μM interleukin 8 (IL-8) for 20 min before the addition of 10 μM forskolin. Termination was terminated after 20 min by removal of the medium and addition of 0.1 M HCl. CAMP levels were determined using the CAMP immunoassay system described above.

Data Analysis—Data were representative of at least three independent experiments. Results are expressed as the mean ± S.E. Student’s t-tests were used to determine significant differences (two-tail p < 0.05).

RESULTS

Interaction of RACK1 with G Proteins in Vivo—We showed previously that RACK1 not only binds purified retinal Gβγ, but also Gαo,β1γ1 in vitro (14). To determine if these observations could be extended to other G protein subunits in vivo, we co-expressed Gβγ2 with GST or GST-RACK1 in COS-7 cells, and precipitated it from cell lysates using glutathione-Sepharose beads. As shown in Fig. 1A, Gβγ was detected in the precipitate containing GST-RACK1, but not GST, indicating a specific association of RACK1 with Gβγ. Intriguingly, when Gβγ was co-expressed with Gα2, Gαo, Gαq, or Gα12, binding of Gβγ to RACK1 was significantly inhibited (Fig. 1, B–D). Co-immunoprecipitation studies indicated that the co-expressed Gα subunits form heterotrimers with Gβγ (data not shown). This suggests that heterotrimeric G proteins do not bind or have lower binding affinity to RACK1. In support of this contention, when heterotrimeric G protein were activated to release Gβγ subunits by co-expressed receptors, the αoA, αq, and β2-ARs, respectively, the association of Gβγ with GST-RACK1 was restored (Fig. 1, B–D). However, no binding of Gα subunits to RACK1 was detected despite their significant expression in cell lysates (Fig. 1, B–D). These results indicate that RACK1 binds only the free Gβγ subunit but not the heterotrimetric G protein nor the Gα subunit in vivo.

Gβγ Targets RACK1 to Cell Membranes—We next examined if interaction with Gβγ alters the cellular distribution of RACK1. As shown in Fig. 2A (panels a and b), in the absence of overexpressed Gβγ, endogenous RACK1 and RACK1-V5-His were evenly distributed in the cytosol of COS-7 cells. In con-
**Regulation of Gβγ Functions by RACK1**

**Fig. 1. Association of RACK1 with Gβγ**. A, Gβγ was co-precipitated with GST-RACK1 from COS-7 cells. COS cells were transiently transfected with Gβ1 and Gγ2, plus GST (lane 1) or GST-RACK1 (lane 2) cDNAs. After cross-linking with DSP (2 mM), cells were lysed and the proteins were precipitated using glutathione-Sepharose beads. After resolution by SDS-PAGE, the protein complexes were visualized by immunoblotting with an anti-Gβ (upper panel) or anti-RACK1 (lower panel) antibody. B-D, GST-RACK1 binds free Gβγ released from heterotrimeric Gα, Gβ, and Gγ. COS-7 cells transiently expressing GST-RACK1 with α2-AR and Gγβγ (B), with α12-AR and Gαγβγ (C), or with α2-AR and Gγβγ (D) were stimulated with (-)-epinephrine (Epi, 10 μM) for the indicated time (B), (-)-epinephrine (10 μM) (C), or (-)-isoproterenol (ISO, 10 μM) (D) for 20 min. GST-RACK1 was then precipitated as described above. GST-RACK1, Gβγ, and the Gα subunits were detected using specific antibodies. The expression of Gβγ and the Gα subunits in cell lysates was also shown.

**Fig. 2. Gβγ targets RACK1 to membranes.** A, co-expression with Gβ1γ2 promotes the association of RACK1-V5-His with the plasma membranes. The intracellular localization of endogenous RACK1 (a), transiently expressed RACK1-V5-His (b), or RACK1-V5-His plus YFP-β1γ2 (c and d) in COS-7 cells was investigated by immunostaining using primary monoclonal antibodies against RACK1 (a) or V5 epitope (b and d) and a secondary Alexa 568-conjugated anti-mouse antibody, and then analyzed using confocal microscopy. Panel e displays an overlay of the signals from panels c and d. B, Gβγ enhances the association of MBP-RACK1 with lipid vesicles. MBP or MBP-RACK1 (30 pmol) in the absence (Control) or presence of Gβ1γ2 or Gβ1γ2 (10 pmol) was incubated with lipid vesicles for 60 min on ice, followed by centrifugation to separate bound and unbound MBP fusion proteins. After SDS-PAGE fractionation, the distribution of the MBP fusion proteins between the supernatant (S, open bars) and the pellet (P, filled bars) was visualized by immunoblotting with an anti-MBP antibody. *, significant differences (p < 0.05) versus control in the absence of Gβγ. A representative Western blot is also displayed.

Contrast, a majority of YFP-Gβ1γ2 expressed in COS-7 cells was located in the cell membranes, although a small fraction was also detected in the cytosol (Fig. 2A, panel c). When YFP-Gβ1γ2 was co-expressed with RACK1-V5-His, the cellular distribution of YFP-Gβ1γ2 was not significantly altered (Fig. 2A, panels c–e). However, it resulted in a punctate distribution of RACK1-V5-His both in the cell membranes and in the cytosol where it co-localized with YFP-Gβ1 (Fig. 2A, panels c–e). These results suggest that Gβγ may target RACK1 to the cell membranes. To confirm these findings, we further assessed the effect of Gβγ on the association of an MBP-RACK1 fusion protein with lipid vesicles in vitro. As shown in Fig. 2B, after incubation with the lipid vesicles followed by centrifugation to separate the bound from the free MBP-RACK1, MBP-RACK1 was found in both the supernatant (45%) and the lipid membrane (55%). The association of MBP-RACK1 with the lipid membrane is most likely due to MBP, because MBP alone was predominantly associated with the lipid (75%) in the lipid membranes. Notably, the addition of either Gβ1γ2 or Gβ1γ2 to the lipid vesicles significantly enhanced the association of MBP-RACK1 with the lipid (70 and 80% for β1γ2 and β1γ2, respectively), although it has little effect on MBP alone (Fig. 2B). Taken together, these findings suggest that the physical interaction between Gβγ and RACK1 facilitates the translocation of RACK1 from the cytosol to the membrane.

**RACK1 Inhibits Specific Gβγ Functions**—Because RACK1 binds to Gβγ in cell membranes where the interaction of Gβγ with effectors occurs, we questioned if RACK1 could affect the ability of Gβγ to activate effectors such as PLCβ2. As shown in Fig. 3A, co-expression of Gβ1γ2 with PLCβ2 in COS-7 cells resulted in a 2- to 3-fold increase in inositol phosphate (IP) accumulation. Co-expression of GST-RACK1 had little effect on the basal IP turnover but significantly inhibited the Gβ1γ2-mediated IP signaling. This effect was specific to RACK1, because co-expression with GST had no effect on the Gβ1γ2-stimulated IP generation (Fig. 3A). Moreover, like GST-RACK1, overexpression of RACK1-V5-His inhibited Gβ1γ2-mediated PLCβ2 activation (data not shown). The magnitude of the inhibitory effect of GST-RACK1 depended on the level of its expression (data not shown). The transfection of 4 μg of GST-RACK1 plasmid relative to 1 μg of Gβ1γ2 plasmid inhibited Gβ1γ2-mediated IP accumulation by ~50% (Fig. 3A).

We then evaluated if RACK1 could interfere with receptor-
mediated PLC activation through endogenous G\(\beta\gamma\). The \(\alpha_{2\alpha}\)-AR was chosen for this study, because it was known to activate phosphatidylinositol (PI) hydrolysis through the G\(\beta\gamma\) released from the activated heterotrimer G. As reported previously (22, 24) and shown in Fig. 3B, stimulation of COS-7 cells transiently expressing the \(\alpha_{2\alpha}\)-AR with the \(\alpha_{2\alpha}\)-AR agonist UK14,304, resulted in a small but significant increase in IP accumulation, which was completely abolished by pertussis toxin pre-treatment. Co-expression of GST-RACK1 blocked the \(\alpha_{2\alpha}\)-AR-mediated IP response (Fig. 3B). Similarly, the IP response initiated by the chemokine receptor CXCR2 transiently expressed in COS-7 cells via the pertussis toxin-sensitive pathway was also inhibited by RACK1 (data not shown).

Because RACK1 can interact with many target proteins other than G proteins, it is possible that the inhibition of G\(\beta\gamma\) signaling by RACK1 was mediated indirectly through the perturbation of other signaling pathways regulated by RACK1. To evaluate this possibility, the effect of RACK1 on G\(\beta\gamma\)-mediated PLC\(\beta2\) stimulation was further evaluated in vitro using heterologously expressed and purified proteins. As shown in Fig. 3C, 10 \(\mu\)M MBP-RACK1 specifically inhibited the G\(\beta_1\gamma_2\) but not the Ca\(^{2+}\)-mediated increase in \(^{3}H\)-labeled phosphatidylinositol 4,5-biphosphate hydrolysis. The inhibitory effect of MBP-RACK1 on G\(\beta_1\gamma_2\) signaling was dose-dependent, with an IC\(_{50}\) of about 4.9 \(\mu\)M (Fig. 3D). This effect was specific, because neither G\(\beta_1\gamma_2\) nor Ca\(^{2+}\)-mediated PLC\(\beta2\) stimulation was affected by MBP alone. Moreover, because MBP-RACK1 did not affect Ca\(^{2+}\)-mediated PLC activation, the inhibition of G\(\beta_1\gamma_2\) signaling is unlikely due to a direct inhibition of PLC enzyme activity. Therefore, the inhibition of RACK1 on G\(\beta\gamma\) signaling is directly related to its binding to G\(\beta\gamma\).

To determine if RACK1 functions as a general inhibitor of G\(\beta\gamma\)-dependent signaling processes, we evaluated the effect of MBP-RACK1 on G\(\beta\gamma\)-mediated ACII activation. As shown in Fig. 4A, the activity of baculovirus-expressed ACII in Sf9 cell membranes was stimulated 100- and 150-fold by GTP\(\gamma\)S-bound Ga\(_{16}\) (10 nM) and forskolin (10 \(\mu\)M), respectively. G\(\beta_1\gamma_2\) (50 nM) itself had little effect on the activity of ACII (data not shown), but it enhanced Ga\(_{16}\)-mediated ACII activation by 2.5-fold (Fig. 4A). 10 \(\mu\)M MBP-RACK1 inhibited basal ACII activity by 50% and G\(\beta_1\gamma_2\)-mediated stimulation by 80% but had little effect on forskolin- or Ga\(_{16}\)-stimulated activity. At the same concentration, MBP did not affect the basal activity of ACII, but caused about 40% inhibition of the G\(\beta_1\gamma_2\)-mediated stimulation (Fig. 4A).
stimulation in a dose-dependent manner (Fig. 4). Notably, the filled bars /H9252, /H9262 ACII were assayed for the activity of ACII in the absence (Control) or membranes (1 μg). The activity of ACII was measured in the absence (open bars, Basal) or presence of forskolin (10 μM) (hatched bars, Forskolin), GTP-S-bound Goi (10 nM) (cross bars, Goi) or GTPyS-bound Goi plus Gβ1γ2 (50 nM) (filled bars, βγ1γ2). * p < 0.05 versus the activity of ACII in the absence of MBP or MBP-RACK1. B, Gβ1γ2 (50 nM) was preincubated with increasing concentrations of MBP (□) or MBP-RACK1 (○) for 30 min on ice before adding into a reaction containing 10 nM of GTP-S-bound Goi and 1 μg of cell membranes containing ACII. ACII activity was measured as described above. *, p < 0.05 versus the activity of ACII in the presence of MBP.

Fig. 4. RACK1 inhibits Gβ1γ2-mediated ACII activation. A, cell membranes (1 μg of total protein) prepared from Sf9 cells expressing ACII were assayed for the activity of ACII in the absence (Control) or presence of MBP (10 μM) or MBP-RACK1 (10 μM) for 20 min at 30 °C. The activity of ACII was measured in the absence (open bars, Basal) or presence of forskolin (10 μM) (hatched bars, Forskolin), GTP-S-bound Goi (10 nM) (cross bars, Goi) or GTPγS-bound Goi plus Gβ1γ2 (50 nM) (filled bars, βγ1γ2). * p < 0.05 versus the activity of ACII in the absence of MBP or MBP-RACK1. B, Gβ1γ2 (50 nM) was preincubated with increasing concentrations of MBP (□) or MBP-RACK1 (○) for 30 min on ice before adding into a reaction containing 10 nM of GTP-S-bound Goi and 1 μg of cell membranes containing ACII. ACII activity was measured as described above. *, p < 0.05 versus the activity of ACII in the presence of MBP.

4A). These data suggest that MBP may have nonspecific effects on the Gβ1γ2-mediated ACII activation. However, because the effect of MBP-RACK1 was significantly greater than that of MBP alone, the additional inhibition of the Gβ1γ2-mediated stimulation by MBP-RACK1 is likely specific to RACK1. The reason that MBP-RACK1 also inhibited the basal activity of ACII is unknown, but it seems to be specific to RACK1, because MBP did not have an effect. Moreover, it is unlikely that MBP-RACK1 directly inhibited the enzyme activity of ACII, because the activity stimulated by forskolin or Goi was unaffected by MBP-RACK1 (Fig. 4A). Additional studies indicate that, as with the inhibition of the Gβ1γ2-mediated PLCβ2 activation, MBP-RACK1 abolished the Gβ1γ2-mediated ACII stimulation in a dose-dependent manner (Fig. 4B). Notably, the IC50 of MBP-RACK1 for inhibiting Gβ1γ2-mediated ACII activation (2.5 μM) was close to that for inhibiting PLCβ2 (4.9 μM), suggesting that inhibition occurs through a similar mechanism.

To determine if other Gβγ functions were inhibited by RACK1, we tested the α2AR-AR-mediated MAPK activation in HEK293 cells. As shown in Fig. 5 (A and B), activation of the α2AR induced phosphorylation of p42 and p44 MAPKs in a dose-dependent manner. These responses were largely abolished by pertussis toxin pre-treatment or co-transfection with Goi, suggesting that they were mediated by Gβγ. However, despite a 2-fold increase over the level of the endogenous RACK1, overexpression of GST-RACK1 did not affect the α2AR-AR-initiated MAPK activation. Similarly, overexpression of RACK1 does not affect the α2AR-AR-mediated MAPK activation in COS-7 cells (data not shown). In addition, similar results were obtained when the CXCR2-mediated MAPK activation was examined (data not shown).

We also evaluated the consequence of GST-RACK1 overexpression on chemotaxis of HEK293 cells transiently expressing CXCR2, a response that was mediated by Gβγ. As shown in Fig. 4C, IL-8 induced chemotaxis of HEK293 cells expressing the CXCR2, which was inhibited by co-expressed Goi, supporting the involvement of Gβγ. Overexpression of GST-RACK1 at a level similar to that shown in Fig. 5A, however, had no effect on CXCR2-induced chemotaxis. These data suggest that RACK1 regulates specific Gβγ functions.

RACK1 Does Not Affect Goi, Gβγ, or Goα-mediated Signaling—To further explore the specificity of RACK1 on G protein signal transduction, we evaluated the effect of RACK1 on signaling mediated by the Goi, Gβγ, and Goα subunits. For evaluating the Goi signaling pathway, we transiently expressed the α1A-AR or the constitutively active Goα,Q209L mutant in COS-7 cells. As seen in Fig. 6 (A and B), activation of the α1A-AR resulted in Goi-mediated PLC activation, whereas overexpression of the constitutively active Goα,Q209L mutant caused an increase in basal IP production. Neither the α1A-AR nor the Goα,Q209L mutant-mediated IP generation was affected by GST-RACK1 when it was expressed at a level that was not significantly different from that seen in Fig. 3A. To evaluate the effect of RACK1 on cAMP signaling mediated by the Goi or Goα subunit, we used HEK293 cells that stably express the chemokine receptor CXCR2 and endogenously express the β2-AR. Stimulation of the cells with forskolin resulted in a marked increase in cAMP levels (Fig. 6C). Pretreatment with the CXCR2 agonist IL-8, inhibited the forskolin-stimulated cAMP accumulation by 60% through the activation of Goi (Fig. 6C). As compared with the GST expression control, overexpression of either GST-RACK1 or RACK1-V5-His had no effect on the inhibition of cAMP accumulation by CXCR2. Similarly, overexpression of GST-RACK1 or RACK1-V5-His did not affect the β2-AR-stimulated increase in cAMP production (Fig. 6C). The inability of RACK1 to affect the β2-AR- and CXCR2-mediated cAMP signaling is not due to its expression level, because it was expressed at a 2-fold increase over the level of the endogenous RACK1. Taken together, these data indicate that RACK1 does not interfere with signaling through Goα subunits.

DISCUSSION

RACK1 was first implicated in signal transduction when it was found to stabilize PKC in the active state and aid in its translocation to specific cellular fractions (15). Recent studies indicate that RACK1 also participates in the regulation of other signaling pathways, including interferon receptor-mediated activation of signal transducer and activator of transcription 1 (25), insulin-like growth factor-dependent signaling (26, 27), and Src activation (28, 29). Here we provide evidence for a new role of RACK1 in the regulation of G protein signaling mediated specifically by Gβγ.

Our data show that overexpression of RACK1 selectively inhibits both Gβj-coupled receptor-stimulated and direct Gβγ-mediated PLC signaling but not that mediated by Goα or by receptors that act through Goq. Furthermore, RACK1 inhibits βγ-mediated ACII activation, but not cAMP signaling mediated by receptors acting through Goα or Goq. These findings indicate that RACK1 is a specific regulator of Gβγ signaling. In line with the role of RACK1 in regulating Gβγ signaling, RACK1 was found to bind only free Gβγ subunits liberated from G protein activation, but not Ga subunits nor heterotrimeric G proteins. In particular, Gβγ can recruit RACK1 from the cytosol to the membrane where Gβγ-mediated signal transduction occurs. The ability of RACK1 to discriminate Goα and Gβγ subunits is consistent with our previous findings that RACK1...
FIG. 5. RACK1 does not affect Gβγ-mediated MAPK activation and chemotaxis of HEK293 cells via CXCR2. A, HEK293 cells transiently transfected with α2A-AR together with GST, GST-RACK1, or Gαt DNAs as indicated were pretreated with (lane 4) or without (lanes 1–3) pertussis toxin (1 μg/ml) overnight. The cells were then stimulated without (lane 1) or with 0.1 μM (lane 2) or 10 μM (lanes 3 and 4) of (-)-epinephrine for 5 min. Phosphorylation of p42/44 MAPK was detected by Western blotting (upper panel). Parallel blots were probed with p42/44 MAPK antibody to monitor equal loading of MAPKs (middle panel), and RACK1 antibody to confirm expression of GST-RACK1 (lower panel). B, the density of phospho-p42/44 bands was determined by densitometric scanning. Data were expressed as -fold increase over the basal. C, HEK293 cells were transiently transfected with CXCR2 together with GST, GST-RACK1, or Gαt cDNAs. Chemotaxis in response to IL-8 (1–250 ng/ml) stimulation was performed as described under “Experimental Procedures.”
binds the Gβγγ1 but not the Goq subunit in vitro (14). Although RACK1 was shown to bind heterotrimeric Gs in vitro (14), it does not seem to interact with other classes of heterotrimeric G proteins in vivo. The reason for the differential binding of RACK1 to different classes of G proteins is unknown but may stem from the difference in the structure of Goq subunits or the composition of Gγ subunits. However, our in vitro GST-pull-down studies did not reveal any difference in the binding of GST-RACK1 to either Gβγγ1 or Gβγγ2 (data not shown), suggesting that Gγ subunits do not play a major role in the interaction of Gβγ with RACK1.

Because RACK1 is a scaffolding protein, theoretically it can recruit other proteins to indirectly modulate the function of Gβγ. However, our findings that RACK1 also inhibits Gβγ-mediated effector activation in in vitro assays that consist of purified proteins without the presence of other RACK1 partners, indicate that the binding of RACK1 to Gβγ itself is sufficient to modulate the interaction of Gβγ with effectors. Because conformational changes on Gβγ are not required for its activity, binding of RACK1 likely sterically hinders the access of effector proteins to Gβγ. However, we cannot exclude the possibility that other RACK1-binding proteins may play a role in regulating Gβγ function in vivo. Indeed, because PKC-mediated phosphorylation can regulate the activity of many Gβγ effectors, such as Ca2+ channels (30), PLCβ (31, 32), and AC (33), it is conceivable that RACK1 might regulate Gβγ signaling via modulating the activity of PKC in cells. However, we found that the addition of PKC inhibitors immediately after transfection did not affect the ability of RACK1 to inhibit Gβγ-mediated PLCβ2 activation (data not shown), suggesting that inhibition of Gβγ signaling by RACK1 does not require PKC activation.

Remarkably, RACK1 appears to regulate only a subset of Gβγ functions. Thus it inhibits Gβγ-mediated PLCβ2 and ACII activation, but not MAPK signaling and chemotaxis of HEK293 cell via CXCR2. Although the effector proteins that are directly regulated by Gβγ to mediate MAPK activation and chemotactic response of CXCR2 in HEK293 cells are not fully elucidated, both responses probably involve phosphoinositide 3-kinase γ (23, 34). Previous studies indicate that the functional domains on Gβγ for regulation of different effectors are overlapping but not identical (17). A simple explanation for the ability of RACK1 to discriminate different Gβγ effectors may be due to the binding of RACK1 to a unique contact region of Gβγ that is critical for activation of some but not other effectors. An alternative explanation is that RACK1 may have differential activities to different Gβγ dimers, which likely co-exist in COS-7 and HEK293 cells and may regulate distinct effectors. However, we have evidence that RACK1 can interact with multiple isoforms of Gβγ with similar binding affinities (data not shown). In addition, we found that overexpression of RACK1 inhibits the cGαA-AR-mediated PLCβ activation but has no effect on the activity of MAPK stimulated by the cGαA-AR in COS-7 cells. The activation of both PLCβ and MAPK by the cGαA-AR is mediated through Gβγ released from Goa. Although specific Gβγ pairs have been reported to couple to particular receptors and Goa subunits, there is not evidence that the same receptor uses different Gβγ dimers to stimulate particular effectors (33, 36). Thus, the ability of RACK1 to distinguish different effectors of Gβγ is unlikely due to its differential interactions with different Gβγ dimers. This characteristic of RACK1 is different from those of several recently described Gβγ-binding proteins, including phosducin, PhLPs, and GRKs (8, 10, 37). These proteins interact with the Goa-binding regions of Gβγ and inhibit all known aspects of Gβγ functions.

The unique ability of RACK1 to differentially regulate different Gβγ functions may have important implications for signaling specificity of Gβγ. Gβγ becomes activated when it is released from activated heterotrimeric G proteins, and it has a long list of effectors, including many ion channels and enzymes, such as PLC, AC, phospholipase A2, phosphoinositide 3-kinase, Bruton and T cell-specific kinase tyrosine kinases, and select members of the GRK family (38). In addition, Gβγ interacts with many other proteins, including dynamin-1 (39), ADP-ribosylation factor (40), calmodulin (41), phosphoducin (9), PhLPs (10), syntaxin 1A and SNAP25 (42), and pleckstrin homology domain-containing proteins (40). However, it is clear that not
all of G\(\beta\)\(y\) effects are activated under a given stimulus, although what constraining factors contribute to the signaling specificity of G\(\beta\)\(y\) remains elusive. The ability of RACK1 to inhibit specific functions of G\(\beta\)\(y\) suggests that it may contribute to signaling specificity of G\(\beta\)\(y\) by localizing particular G\(\beta\)\(y\) effectors within signaling complexes. Studies are currently underway to address these hypotheses.

Acknowledgments—We thank Drs. Robert M. Graham for various clones, Alan Smrcka (University of Rochester, Rochester, NY) for PLC\(\beta\) baculovirus and purified proteins, Ravi Iyengar (Mount Sinai School of Medicine, NY) for AChI baculovirus, Richard J. Miller (University of Chicago) for PEYFP-\(\beta\)1, and Drs. Ann Richmond, Bryan D. Spiegelberg, and Cheryl Bartleson for critical reading of the manuscript.

REFERENCES

1. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
2. Whiteway, M. S., Wu, C., Leeuw, T., Clark, K., Fourest-Lieuvin, A., Thomas, D. Y., and Leberer, E. (1995) Science 269, 1572–1575
3. Neptune, E. R., and Bourne, H. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14489–14494
4. Wu, L., Valkema, R., Van Haastert, P. J., and Devreotes, P. N. (1995) J. Cell Biol. 129, 1607–1617
5. Wu, L., Valkema, R., Van Haastert, P. J., and Devreotes, P. N. (1995) J. Cell Biol. 129, 1607–1617
6. Pecrin, M., Welsch, D. W., Westphal, M., Schwartz, J. M., Wu, L., Brasco, E., Gerisch, G., Devreotes, P., and Bozzaro, S. (1998) J. Cell Biol. 141, 1529–1537
7. Iaccarino, G., and Koch, W. J. (1999) IURMB Life 48, 257–261
8. Schulz, R. (2001) Pharmacol. Res. 43, 1–10
9. Bauer, P. H., Muller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E. J., and Lohse, M. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2100–2104
10. McLaughlin, J. N., Thulin, C. D., Bray, S. M., Martin, M. M., Elton, T. S., and MacIntosh, P. W. (2002) J. Biol. Chem. 277, 31485–31495
11. Hawes, B. E., Touhara, K., Kurone, H., Leffkowitz, R. J., and Inglese, J. (1994) J. Biol. Chem. 269, 28625–28630
12. Inglese, J., Luttrell, L. M., Iniguez-Lluhi, J. A., Touhara, K., Koch, W. J., and Leffkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3637–3641
13. Dell, E. J., Conner, J., Chen, S., Stebbins, E. G., Skiba, N. P., Mochly-Rosen, D., and Hamm, H. E. (2002) J. Biol. Chem. 277, 49888–49895
14. Mazzoni, M. R., Malinski, J. A., and Hamm, H. E. (1991) J. Biol. Chem. 266, 14072–14081
15. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reveney, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Leffkowitz, R. J., and Hamm, H. E. (1998) Science 280, 1271–1274
16. Lee, E., Linder, M. E., and Gilman, A. G. (1994) Methods Enzymol. 237, 146–164
17. Chen, S., Lin, F., Xu, M., Riek, R. P., Novotny, J., and Graham, R. M. (2002) Biochemistry 41, 6045–6053
18. Pitcher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J., and Leffkowitz, R. J. (1996) J. Biol. Chem. 271, 24907–24913
19. Chen, S., Lin, F., Inoue, S., Lee, K. N., Birckchisler, P. J., and Graham, R. M. (1996) J. Biol. Chem. 271, 32593–32591
20. Chen, S., Lin, F., Xu, M., Hwa, J., and Graham, R. M. (2000) EMBO J. 19, 4265–4271
21. Wang, D., Bai, J., Carter, G., Sachpatzidis, A., Lolis, E., and Richmond, A. (2002) Biochemistry 41, 7100–7107
22. Coteccia, S., Kobilka, B. K., Daniel, K. W., Nolan, R. D., Lapetina, E. Y., Caron, M. G., Leffkowitz, R. J., and Regan, J. W. (1999) J. Biol. Chem. 263, 65–69
23. Usacheva, A., Smith, R., Minshall, R., Baida, G., Seng, S., Croze, E., and Calomoni, O. (2001) J. Biol. Chem. 276, 22948–22953
24. Kryly, P. A., Sani, A., and O’Connor, R. (2002) J. Biol. Chem. 277, 22581–22589
25. Hermanto, U., Zong, C. S., Li, W., and Wang, L. H. (2002) Mol. Cell. Biol. 22, 2345–2365
26. Chang, B. Y., Winckler, M., and Cartwright, C. A. (2001) J. Biol. Chem. 276, 20346–20356
27. Chang, B. Y., Conroy, K. B., Masleeder, E. M., and Cartwright, C. A. (1998) Mol. Cell. Biol. 18, 3245–3256
28. Lee, C. S., Dekker, L. V., and Tinker, A. (2001) J. Physiol. 534, 367–379
29. Litsoch, I. (1997) Biochem. J. 326, 701–707
30. Yue, C., Wu, C., Liu, M., Simon, M. I., and Sanborn, B. M. (2000) J. Biol. Chem. 275, 30220–30225
31. Zimmermann, G., and Taussig, R. (1999) J. Biol. Chem. 274, 21673–21666
32. Lopez-desacsa, M., Crespo, P., Dellicari, P., Gutkind, J. S., and Wetters, K. (1997) Science 275, 394–397
33. Kless, W., Schneekloth, H., Hescheler, J., Schulte, G., and Wittig, B. (1992) Nature 358, 424–426
34. Hwang, J. H., Fraser, I. D., Chai, S., Qin, X. F., and Simon, M. I. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 488–493
35. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Leffkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
36. Chang, B. Y., Winckler, M., and Cartwright, C. A. (2001) J. Biol. Chem. 276, 20346–20356
37. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Leffkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
38. Neer, E. J., and Neer, E. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 167–203
39. Liu, J. P., Yajima, Y., Li, H., Ackland, S., Akiya, Y., Stewart, J., and Kawauchi, S. (1997) Mol. Endocrinol. 13, 61–71
40. Colombo, M. I., Inglese, J., D’Souza-Schorey, C., Beron, W., and Stahl, P. D. (1995) J. Biol. Chem. 270, 24564–24571
41. Liu, M., Yu, B., Nakashashi, O., Wieland, T., and Simon, M. (1997) J. Biol. Chem. 272, 14301–14307
42. Blackmer, T., Larsen, E. C., Takahashi, M., Martin, T. F., Alford, S., and Hamm, H. E. (2001) Science 292, 293–297
