Receptor for Activated C Kinase 1 (RACK1), a novel G\(\beta\gamma\)-interacting protein, selectively inhibits the activation of a subclass of G\(\beta\gamma\) effectors such as phospholipase C \(\beta\) 2 (PLC\(\beta\)2) and adenylyl cyclase II by direct binding to G\(\beta\gamma\) (Chen, S., Dell, E. J., Lin, F., Sai, J., and Hamm, H. E. (2004) J. Biol. Chem. 279, 17861–17868). Here we have mapped the RACK1 binding sites on G\(\beta\gamma\). We found that RACK1 interacts with several different G\(\beta\gamma\) isoforms, including G\(\beta_1\gamma_1\), G\(\beta_1\gamma_2\), and G\(\beta_3\gamma_2\), with similar affinities, suggesting that the conserved residues between G\(\beta_1\) and G\(\beta_3\) may be involved in their binding to RACK1. We have confirmed this hypothesis and shown that several synthetic peptides corresponding to the conserved residues between G\(\beta_1\) and G\(\beta_3\) inhibit the RACK1/G\(\beta\gamma\) interaction as monitored by fluorescence spectroscopy. Interestingly, these peptides are located at one side of G\(\beta_1\) and have little overlap with the G\(\alpha\) subunit binding interface. Additional experiments indicate that the G\(\beta\gamma\) contact residues for RACK1, in particular the positively charged amino acids within residues 44–54 of G\(\beta_1\), are also involved in the interaction with PLC\(\beta\)2 and play a critical role in G\(\beta\gamma\)-mediated PLC\(\beta\)2 activation. These data thus demonstrate that RACK1 can regulate the activity of a G\(\beta\gamma\) effector by competing for its binding to the signal transfer region of G\(\beta\gamma\).

G\(\beta\gamma\) subunits liberated from heterotrimeric G proteins following the activation of G protein-coupled receptors play critical roles in many cellular processes (1, 2). They regulate a variety of effector molecules ranging from enzymes such as phospholipase C \(\beta\) (PLC\(\beta\)) and adenylyl cyclase I to ion channels. The G\(\beta\) subunit has six isoforms, G\(\beta_1\), G\(\beta_2\), G\(\beta_3\), and two G\(\beta\) splice variants, long and short (2). Although G\(\beta\) isoforms 1–4 share more than 85% amino acid identity, G\(\beta_5\) is only about 50% identical to the other isoforms. G\(\beta\) subunits are WD40 repeat proteins whose structures are characterized by the presence of an N-terminal \(\alpha\)-helix and a C-terminal toroidal structure made up of seven-bladed B propellers (6). The unique toroidal B propeller structure defines multiple surfaces including a top, bottom, and outer surface on G\(\beta\) for interactions with receptors, G\(\alpha\) subunits, and effectors. Mutagenesis analyses and co-crystal studies of G\(\beta\gamma\) with interacting proteins such as phosducin and G protein-coupled receptor kinase 2 (GRK2) have identified multiple protein interaction sites on G\(\beta\gamma\) (1–4).

\(\gamma\) The abbreviations used are: PLC\(\beta\), phospholipase C \(\beta\); GTP, guanosine triphosphate; GDP, guanosine diphosphate; Ca\(\text{II}\), calcium; Ca\(\text{II}\)EGTA, calcium-free; [3H]GTP, [\(\gamma\)-3H]GTP; GST, glutathione S-transferase; MBP, maltose-binding protein; RACK1, receptor for activated C kinase 1; GRK, G protein-coupled receptor kinase; ACII, adenylyl cyclase II.

However, residues critical for activation of all known G\(\beta\gamma\) effectors appear to cluster on a surface of G\(\beta\gamma\) that is covered by the G\(\alpha\) subunit in the heterotrimer (5). This explains why dissociation of G\(\beta\gamma\) from G\(\alpha\) is required for the activation of all known effectors. In addition to the G\(\alpha\) contact region, other sites on G\(\beta\) also participate in effector binding and activation. These include the N-terminal coiled-coil domain and the outer surface of the \(\beta\) propeller structure, which may define the signaling specificity of G\(\beta\gamma\) for different effectors (6–8).

G\(\beta\gamma\) has a long list of interacting proteins. In addition to being effectors, a number of interacting proteins can also regulate the activity of G\(\beta\gamma\). These include phosducin, phosducin-like proteins, and GRKs (9, 10). Binding of these proteins to G\(\beta\gamma\) results in inhibition of all known effectors as their binding sites on G\(\beta\gamma\) are shared by the G\(\alpha\) subunits. Recently, we have identified a novel G\(\beta\gamma\)-interacting protein, RACK1, that can also modulate G\(\beta\gamma\) functions (11–13). However, unlike other G\(\beta\gamma\) regulatory proteins, RACK1 only affects the activation of a subset of G\(\beta\gamma\) effectors, such as PLC\(\beta\)2 and ACII, but has no effect on other G\(\beta\gamma\) functions (12). Given the fact that the G\(\alpha\) binding interface contains molecular determinants for all known G\(\beta\gamma\) effectors, these findings suggest that RACK1 may bind to a domain of G\(\beta\gamma\) outside the G\(\alpha\) contact region.

Here we have used a combination of peptide and fluorescence spectroscopic approaches to define the RACK1 binding sites on G\(\beta\gamma\). Moreover, we have evaluated the contribution of the RACK1 contact residues to G\(\beta\gamma\)-mediated PLC\(\beta\)2 activation. The results of this study provide evidence that RACK1 binds to a unique region of G\(\beta\gamma\) critical for its signal transfer function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were purchased from GL Biochem Ltd. (Shanghai, China) and purified to >95% purity by high performance liquid chromatography. [\(\gamma\)-3H]Phosphatidylinositol 4,5-biphosphate (5.5 Ci/mmol) was from PerkinElmer Life Sciences. Mouse anti-RACK1 antibody was from BD Transduction Laboratories, and rabbit anti-G\(\beta\)5 from Upstate Group LLC. Rabbit anti-G\(\beta_5\) was generated by our laboratory using purified G\(\beta_5\gamma_5\) as the immunogen. Nickel-nitrilotriacetic acid-agarose was from Qiagen, amylose resin from New England Biolabs, and glutathione-agarose from Amersham Biosciences. Other chemicals were of the highest grade available commercially.

**Purification of G\(\beta\)γ, G\(\beta_3\)γS959∆PSR, and PLC\(\beta\)2—G\(\beta\)γ was purified from bovine retina as described (14). G\(\beta_3\)γ2, G\(\beta_5\)γ2, G\(\beta\)5γS959∆PSR, and PLC\(\beta\)2 were expressed in S9 cells by infection with baculovirus encoding His\(\text{S}_{1}\)G\(\gamma_2\), G\(\beta_3\)His\(\text{S}_{1}\)G\(\gamma_2\), and G\(\beta\)5His\(\text{S}_{1}\)G\(\gamma_{5}\)∆PSR and GB5, His\(\text{S}_{1}\)-PLC\(\beta\)2, respectively, and purified to near homogeneity using nickel-nitrilotriacetic acid as described (5, 15).

**Purification of Other Proteins**—Maltose-binding protein (MBP) and MBP-RACK1 were expressed in Escherichia coli BL21 cells and purified as described (11).

**GST Pulldown Assays in Vitro**—Binding of G\(\beta\)γ and mutants to glutathione S-transferase (GST) fusion RACK1, the C terminus of GRK2...
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(GRK2-ct), and phosducin in vitro was conducted as described previously (11).

**Fluorescent Labeling of Gβγ**

Gβ1γ1 was labeled with 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt (M8) as described previously (16, 17). Interaction of M8-labeled Gβ1γ1 with MBP-RACK1 or PLCβ2 was monitored using a Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty. Ltd.) with excitation at 322 nm and emission at 420 nm. To determine effects of the Gβ1-derived peptides on the RACK1/Gβγ and PLCβ2/Gβγ interactions, peptides were added to the cuvette containing M8-labeled Gβ1γ1 before the addition of MBP-RACK1 or PLCβ2. All fluorescence measurements were performed at 25 °C in HEPES buffer containing 20 mM HEPES (pH 7.5), 5 mM MgCl2, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. Peptides were prepared as 10–20 mM-stocks in 10 mM HEPES buffer with pH adjusted to near 8.0. Background fluorescence from peptides and MBP-RACK1 in the absence of M8-labeled Gβ1γ1 was corrected in all measurements.

**Measurement of PLCβ2 Activity**—The Gβγ and peptide-mediated PLCβ2 activation was determined essentially as described before (12).

**Construction, Expression, and Purification of Gβγ Mutants**—To construct Gβ1 mutants for expression in S9 cells, a GATEWAY cloning system (Invitrogen) was used. Briefly, bovine Gβ1 was first cloned into pENTR/D/D-TOPO vector (Invitrogen) and site-directed mutagenesis was performed on pENTR/D/D-TOPO Gβ1 using a QuikChange site-directed mutagenesis kit (Stratagene). After the mutant Gβ1 was transferred into a donor plasmid pDEST8 by homologous recombination reactions, recombinant baculoviruses encoding the Gβ1 mutants were generated using a Bac-To-Bac baculovirus expression system (Invitrogen). Expression and purification of mutant Gβ1γ2 from S9 cells were performed as described above.

**Data Analysis**—Unless indicated, data are 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 Different Gβγ Isoforms**—To identify RACK1 binding sites on Gβγ, we first compared the binding of GST-RACK1 to different Gβγ isoforms, including Gβ1γ1, Gβ2γ2, and Gβ5γ2. As shown in Fig. 1, A and B, GST-RACK1 interacts with purified Gβ1γ1, Gβ2γ2, and Gβ5γ2 with similar affinities (0.3–0.5 μM). Gγ1 and Gγ2 share only 37% amino acid identity and are post-translationally modified with different lipids, with Gγ1 being farnesylated and Gγ2 geranylgeranylated. The similar binding of RACK1 to Gβ1γ1 and Gβ2γ2 suggests that Gγ is not a major determinant for the RACK1/Gβγ interaction. Consistent with this hypothesis, after forming a complex with its native partner, RGS9L, Gβγ can bind to GST-RACK1 as well as Gβ5γ2 (data not shown).

**RACK1 Binds to a Unique Region of Gβ—Gβ1 and Gβ3 share only ~50% amino acid identity. However, the conserved residues between these two Gβ isoforms are clustered in the seven blades of Gβ that are known to be involved in interactions with diverse proteins (Fig. 2A). Given the similar binding of Gβ1γ1 and Gβ2γ2 to RACK1, we hypothesized that the RACK1 contact residues on Gβ are localized in these conserved regions. To test this hypothesis, we synthesized a series of peptides corresponding to surface-exposed residues of the conserved regions and determined their effects on the RACK1/Gβγ interaction (Fig. 2B). For the purpose of synthesis, purification, and solubilization, we have kept the length of peptides at 9–20 amino acids. Except for two peptides, p86–105 and p265–275, that could not be dissolved in solution probably due to the high content of hydrophobic amino acids in the sequences, all other peptides were soluble.

To be able to identify peptides that possess low binding affinities to RACK1, we have developed a sensitive and quantitative fluorescence-based assay to monitor the RACK1/Gβγ interaction in solution. In this assay, purified Gβγ was labeled with an environmentally sensitive thiol-reactive fluorescent probe, M8. Binding of purified MBP-RACK1 to M8-labeled Gβγ was monitored by changes in the intensity and emission wavelength of fluorescence. This method allows us to monitor the interaction of RACK1 with Gβγ in real time in solution and evaluate the ability of peptides to perturb the interaction. Because Gβ1γ1 interacts with RACK1 with similar affinity as other Gβγ isoforms, it was used in this study as it can be easily purified from bovine retina. As shown in Fig. 3, A and B, the addition of MBP-RACK1 to M8-labeled Gβ1γ1 perturbed the interaction of Gβγ with M8-alone had little effect. The increase in fluorescence is probably caused by the enhanced hydrophobicity around the fluorescent probe on Gβγ upon RACK1 binding. The RACK1-mediated enhancement of the M8-Gβγ fluorescence was immediate, suggesting the association of these proteins was rapid, i.e., within the mixing time. The binding affinity for the RACK1/Gβ1γ1 interaction determined from this assay (EC50 ~0.5 μM) is similar to that from GST-RACK1 pulldown assays (EC50 ~0.3 μM), suggesting that the modification of Gβ1γ1 with M8 does not perturb its binding to RACK1.

To screen for peptides that inhibit the RACK1/Gβ1γ1 interaction, we preincubated M8–Gβ1γ1 with or without peptides and then compared the fluorescence before and after the addition of MBP-RACK1. Fig. 4A shows the representative data for two synthetic peptides corresponding...
Mapping RACK1 Binding Sites onto Gβγ

FIGURE 2. Structure of the Gβγ subunit. Structure of the Gβ1γ1 subunit showing the conserved residues between the Gβ1 and Gβ2 (A) and the corresponding residues of the peptides used in this study (B). Pink and red colors in panel A indicate the similar and identical residues between Gβ1 and Gβ2, respectively. The structure of the Gβ1γ1 was generated using Swiss-Protein Data Bank viewer from the crystal coordinates of the Gβ1γ1 (27–29).

FIGURE 3. Characterization of the RACK1/Gβγ interaction using fluorescence spectroscopy. A, fluorescent changes of MB-Gβ1γ1 upon the addition of purified MBP or MBP-RACK1 at the indicated concentrations. The fluorescence of MB-Gβ1γ1 (0.1 µM) was monitored at excitation 322 nm and emission 420 nm as described under “Experimental Procedures.” The addition of various reagents is indicated by arrows. B, affinities of MB-Gβ1γ1 for MBP or MBP-RACK1. Relative fluorescent changes of MB-Gβ1γ1 as a function of MBP or MBP-RACK1 concentrations were expressed as percentage increases of the fluorescence of MB-Gβ1γ1 after the addition of MBP or MBP-RACK1. The EC50 for the binding of MB-Gβ1γ1 to MBP-RACK1 is 0.52 ± 0.09 µM.

to residues 44–54 and 86–98 of Gβ1. Other peptides tested include p113–122 and p136–147 from blade 2 of Gβ1, p159–167 and p177–189 from blade 3, p201–209 and p219–231 from blade 4, p243–251 from blade 5, p287–296 from blade 6, p309–316 and p328–337 from blade 7. Results for some of the tested peptides are summarized in Fig. 4B. Peptides p86–105 and p265–275 were excluded from this study as they could not be dissolved in solution unless using Me2SO, which itself tides p86–105 and p265–275 were excluded from this study as they could not be dissolved in solution unless using Me2SO, which itself interferes with the fluorescence of M8-Gβ1γ1. Except for peptides p44–54, p309–316 and p328–337, p86–98 and p287–295, which showed significant inhibitory effects on the RACK1/Gβγ interaction, all other peptides had no effect. The inhibitory effects of the individual peptides on the RACK1/Gβγ interaction ranged from 10 to 70%. When used in combination, peptides p44–54 and p328–337 caused almost complete inhibition of the RACK1/Gβγ interaction, suggesting that they have additive inhibitory effects (data not shown). In addition, these peptides can also cause the dissociation of Gβγ from RACK1 when added after the formation of the Gβγ-RACK1 complex, suggesting that the peptides may perturb the RACK1/Gβγ interaction through competitive binding for RACK1 with Gβγ. Interestingly, when mapped to the crystal structure of Gβ1γ1, the corresponding residues of these inhibitory peptides are clustered at one side of Gβ1 involving blades 1, 6, and 7 (Fig. 4B, inset). Except for residues Lys-89, Ser-98, and Trp-332, which are contained in peptides p86–98 and p328–337, other Gα subunit-interacting residues do not fall within the RACK1 binding sites in Gβγ (Fig. 5A). This is in contrast to other Gβγ-interacting proteins such as GRK2 and phosducin whose binding sites on Gβγ largely overlap with the Gα binding interface (Fig. 5, B and C) (3, 4).

PLCβ2 and RACK1 Share Overlapping Contact Residues on Gβγ—We have shown previously that RACK1 directly inhibits Gβγ-mediated PLCβ2 activation (12). To determine whether this is due to a competitive binding of RACK1 for the PLCβ2 contact residues on Gβγ, we further evaluated effects of the peptides on the PLCβ2/Gβγ interaction. Similar to RACK1, the addition of PLCβ2 caused a dose-dependent increase in the fluorescence of M8-Gβ1γ1 with an EC50 ~1 µM (data not shown). As with the interaction of RACK1 with Gβγ, peptides p44–54, p86–98, p287–296, p309–316, and p328–337 also inhibited the PLCβ2/Gβγ interaction, with p44–54 being most effective (Fig. 6). In addition to these peptides, peptide p177–189 also inhibited the PLCβ2/Gβγ interaction, although it had no effect on the binding of RACK1 to Gβγ. This suggests that the RACK1 and PLCβ2 binding sites on Gβγ are overlapping but not identical.
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FIGURE 4. Effects of Gβ2-derived peptides on the RACK1/Gβγ interaction. A, relative fluorescent changes of M8-Gβ2γ1 (0.1 μM) upon the addition of buffer and peptides p44–54 or p86–98 (0.5 mM) followed by MBP-RACK1 (0.5 μM). Arrows indicate the addition of various reagents to the cuvette while continuously monitoring the emission of the M8-Gβ2γ1, Gβ2γ1, and Gβ2γ1.8, a summary of the effects of the Gβ2-derived peptides on the binding of RACK1 to Gβγ. Effects of peptides (0.5 mM) on increases in the fluorescence of M8-Gβ2γ1,γ1 elicited by MBP-RACK1 (0.5 μM) in the absence of the peptides (CT). * indicates significant differences (p < 0.05) versus MBP-RACK1-mediated relative fluorescent changes in the absence of the peptides. The localization of the peptides in the blades of Gβ2 is indicated in the figure. Inset, ribbon diagram of Gβ2γ1 structure showing the localization of the corresponding residues of the peptides that inhibit the interaction of RACK1 with M8-Gβ2γ1.

The Gβγ Contact Region for RACK1 Is Critical for PLCβ2 Activation—Based on the ability of Gβ-derived peptides to either perturb Gβγ-mediated PLCβ2 activation or activate PLCβ2 in the absence of Gβγ, Buck and Iyengar (18) have proposed that contact regions of Gβγ for an effector can be involved in either general binding or signal transfer functions. To determine the role of RACK1 binding sites in Gβγ-mediated PLCβ2 activation, we then evaluated effects of the Gβγ-derived peptides on the activity of PLCβ2. As shown in Fig. 7A, a majority of peptides, including those that can perturb the Gβγ/RACK1 and Gβγ/PLCβ2 interactions, did not affect either the basal or the Gβγ-mediated PLCβ2 activation. However, peptide p177–189, which inhibited the interaction of Gβγ with PLCβ2 but not RACK1, abolished Gβγ-stimulated PLCβ2 activity even better than GRK2-ct, suggesting that the corresponding residues of this peptide are involved in binding and signaling of Gβγ to PLCβ2. Interestingly, peptides p44–54 and p86–105 stimulated PLCβ2 activity even in the absence of Gβγ (Fig. 7B). These results are similar to previous reports using peptides from the same region (18, 19), suggesting that the peptides are derived from regions of Gβγ involved in signal transduction. Peptide p86–105 seems to have a more potent ability to stimulate PLCβ2 than p44–54 (Fig. 7B). However, the potency and efficacy of p86–105 could not be accurately obtained in these studies because at higher concentration it precipitated out of solution.

The Charged Amino Acids within Residues 44–54 Are Critical for Efficient Gβγ/RACK1 Interaction and PLCβ2 Activation—Because p44–54 is the most effective peptide in perturbing the interaction of Gβγ with either RACK1 or PLCβ2 and it can activate PLCβ2, we further determined the critical residues mediating its activity. We initially synthesized a series of p44–54 mutant peptides with 2–3 alanine substitutions in each mutant and then determined their effects on the RACK1/Gβγ and PLCβ2/Gβγ interactions (Fig. 8A). As shown in Fig. 8B, except for the mutant A53–54, which showed a reduced inhibitory effect (50 versus 70% for the original peptide) on the interaction of Gβγ with RACK1, but not PLCβ2, all other mutant peptides have decreased abilities to inhibit both Gβγ/RACK1 and Gβγ/PLCβ2 interactions. Their inhibitory effects on the Gβγ/RACK1 and Gβγ/PLCβ2 interactions were reduced from 70 and 60% for the original peptide to 10–20% and 20–30%, respectively. The fact that substitution of residues 53–54 dif-
mutations of Arg-48 and Arg-49 or His-54 (data not shown) did not affect the binding of Gβγ to RACK1. The mutant Gβγ(5A)γ2 showed a slight decrease in affinity but not binding capacity (B_max) for RACK1, suggesting that the 5 charged residues Arg-46, Arg-48, Arg-49, Arg-52, and His-54 contribute to the tight association of Gβγ (Fig. 8B). However, when evaluated in PLCβ2 activity assays, all of the Gβγ(5A)γ2 mutants, Gβγ(R48R49A)γ2, Gβγ(H54A)γ2, and Gβγ(5A)γ2γ2 showed decreases in the maximal stimulation of PLCβ2 activity, with E_max being 50–80% of the wild-type (Fig. 10). However, they displayed different potencies in stimulating PLCβ2. Whereas the potency of Gβγ(5A)γ2 was decreased ~2-fold (EC50 = 85 versus 40 nM for the wild-type), mutants Gβγ(R48R49A)γ2 and Gβγ(H54A)γ2 displayed ~4–5-fold increases in potency (EC50 = 7.7 or 8.5 nM for Gβγ(R48R49A)γ2 or Gβγ(H54A)γ2). These findings suggest that although they are equally important for

To further characterize the overall contribution of the charged amino acids within residues 44–54 to the RACK1/Gβγ interaction and PLCβ2 activation in the context of full-length Gβ, we constructed Gβ mutants with residues Arg-48 and Arg-49, His-54, or all five charged residues Arg-46, Arg-48, Arg-49, Arg-52, and His-54 substituted with alanines. After these mutants were expressed together with His6-Gγ from Sf9 cells and purified to near homogeneity, their abilities to interact with RACK1 and to activate PLCβ2 were evaluated. As shown in Fig. 9, A and B, mutations of Arg-48 and Arg-49 or His-54 (data not shown) did not affect the binding of Gβγ to RACK1. The mutant Gβγ(5A)γ2 showed a slight decrease in affinity but not binding capacity (B_max) for RACK1, suggesting that the 5 charged residues Arg-46, Arg-48, Arg-49, Arg-52, and His-54 contribute to the tight association of Gβγ with RACK1. To ensure that the reduced binding affinity of the mutant Gβγ(5A)γ2 to RACK1 is not secondary to global structural changes, we have also evaluated the binding of this mutant to the C terminus of GRK2 and phosphoducin. Based on the crystal structures of the Gβγγ2-GRK2 and Gβγγ2-phosphoducin complexes, none of the 5 residues is involved in interaction with GRK2 but Arg-42 and Arg-46 are engaged in direct contacts with the C-terminal domain of phosphoducin (3, 4). As expected, the mutant Gβγ(5A)γ2 interacts with GRK2-cct as well as the wild-type Gβγγ2 (Fig. 9C). By contrast, Gβγ(5A)γ2 showed a reduced binding to phosphoducin (Fig. 9D). These findings suggest that mutations of the 5 charged residues did not result in misfolding of Gβγγ2.

When evaluated in PLCβ2 activity assays, all of the Gβγ mutants, Gβγ(R48R49A)γ2, Gβγ(H54A)γ2, and Gβγ(5A)γ2γ2 showed decreases in the maximal stimulation of PLCβ2 activity, with E_max being 50–80% of the wild-type (Fig. 10). However, they displayed different potencies in stimulating PLCβ2. Whereas the potency of Gβγ(5A)γ2 was decreased ~2-fold (EC50 = 85 versus 40 nM for the wild-type), mutants Gβγ(R48R49A)γ2 and Gβγ(H54A)γ2 displayed ~4–5-fold increases in potency (EC50 = 7.7 or 8.5 nM for Gβγ(R48R49A)γ2 or Gβγ(H54A)γ2). These findings suggest that although they are equally important for
maximal stimulation of PLCβ2, the 5 charged residues may play different and even antagonistic roles in the tight association of Gβγ with PLCβ2. These results are consistent with previous reports that a residue in Gβγ may play a different role in the association and signal transduction of Gβγ to effectors (18, 20).

**DISCUSSION**

In this study, we have identified the key contact region of Gβγ for RACK1. Moreover, we have shown that the Gβγ binding sites for RACK1 are shared by PLCβ2 and contribute to Gβγ-mediated PLCβ2 activation. These findings thus provide direct evidence for the ability of RACK1 to compete for a common effector binding site on Gβγ and inhibit Gβγ-mediated effector activation.

Our identification of the RACK1 binding sites on Gβγ was facilitated by the use of a combination of peptide and spectrofluorometric approaches. The use of peptides to compete for the binding of Gβγ to RACK1 allows us to screen a large surface area of Gβγ in a short period. Moreover, it avoids potential structural changes of Gβγ when mutagenesis experiments are performed on Gβγ to identify the RACK1 contact residues. The caveat of this approach, however, is that the binding affinities of the interaction of RACK1 with wild-type and mutant Gβγ were resolved by SDS-PAGE and probed with antibodies against Gβ (upper panel) or GST (middle panel). One percent of Gβγ inputs were also examined by immunoblotting to show the equal loading of wild-type and mutant Gβ1, γ2 (lower panel). B, affinities of the interaction of RACK1 with wild-type and mutant Gβγ. The amount of Gβγ bound to GST-RACK1 was determined by densitometric scanning and calculated using the known amount of purified Gβγ, γ2, as a standard. The EC50 and Bmax are 1.23 ± 0.7 μM and 1.71 ± 0.5 pmol for wild-type Gβ1, γ2, 0.85 ± 0.3 μM and 1.36 ± 0.5 pmol for Gβ1(R48/49A), γ2, and 6.4 ± 3.2 μM and 1.38 ± 0.6 pmol for Gβ1(SA), γ2. C and D, binding of GST-GRK2-ct (C) or GST-phosducin to wild-type Gβ1, γ2, and its mutant Gβ1(SA), γ2. 20–50 pmol of GST-GRK2-ct and GST-phosducin and the indicated concentrations of wild-type and mutant Gβ1, γ2 were used in the GST binding assays. Lanes 1 in panels C and D, GST control.

FIGURE 8. Effects of peptide p44–54 and its mutants on RACK1/Gβγ and PLCβ2/Gβγ interactions and PLCβ2 activity. A, the amino acid sequences of peptide p44–54 and its alanine substitution mutants. The number above the sequence indicates the position of the charged residues. The alanine substitutions in each mutant peptide are underlined. B, effects of alanine substitutions on the ability of the peptide p44–54 to inhibit RACK1/Gβγ and PLCβ2 interactions. The interactions were monitored by fluorescence spectroscopy as described in Fig. 4. Concentrations of peptides (0.5 mM), MBP-RACK1 (0.5 μM), PLCβ2 (0.5 μM), and M8-Gβ1, γ1 (0.1 μM) were used in the assay. Data are presented as percentage inhibition of MBP-RACK1- or PLCβ2-induced relative fluorescent changes of M8-Gβ1, γ1. * indicates significant differences (p < 0.05) versus the inhibition mediated by the original peptide p44–54. C, the activity of purified PLCβ2 (50 ng) in the presence of increasing concentration of peptide p44–54 and its mutants. Data are presented as percentage of the maximal activity of PLCβ2 stimulated by wild-type p44–54.

FIGURE 9. Interactions of wild-type and mutant Gβ1, γ2 with RACK1, GRK2-ct, and phosducin. A, binding of GST-RACK1 (~20 pmol) to purified wild-type Gβ1, γ2, and its mutants Gβ1(R48/49A), γ2, and Gβ1(SA), γ2 at the indicated concentrations. The binding was determined by GST binding assays in vitro. Pellets containing GST-RACK1 and Gβγ were resolved by SDS-PAGE and probed with antibodies against Gβ (upper panel) or GST (middle panel). One percent of Gβγ inputs were also examined by immunoblotting to show the equal loading of wild-type and mutant Gβ1, γ2 (lower panel). B, affinities of the interaction of RACK1 with wild-type and mutant Gβ1, γ2. The amount of Gβγ bound to GST-RACK1 was determined by densitometric scanning and calculated using the known amount of purified Gβγ, γ2, as a standard. The EC50 and Bmax are 1.23 ± 0.7 μM and 1.71 ± 0.5 pmol for wild-type Gβ1, γ2, 0.85 ± 0.3 μM and 1.36 ± 0.5 pmol for Gβ1(R48/49A), γ2, and 6.4 ± 3.2 μM and 1.38 ± 0.6 pmol for Gβ1(SA), γ2. C and D, binding of GST-GRK2-ct (C) or GST-phosducin to wild-type Gβ1, γ2, and its mutant Gβ1(SA), γ2. 20–50 pmol of GST-GRK2-ct and GST-phosducin and the indicated concentrations of wild-type and mutant Gβ1, γ2 were used in the GST binding assays. Lanes 1 in panels C and D, GST control.
ity of the peptides to RACK1 may be significantly lower than when they reside in the native proteins. To circumvent this problem, we have developed a sensitive fluorescence competition approach. This allowed identification of peptides such as p86–98 and p309–316, which have small inhibitory effects, as little as 10%, on the RACK1/Gβγ interaction that may be neglected by using non-equilibrium approaches such as GST pulldown assays. It is noteworthy that of 13 peptides we studied, only 5 (p44–54, p86–98, p287–298, p309–316, and p328–337) significantly inhibit the Gβγ/RACK1 interaction, suggesting that the corresponding residues of these peptides are specifically involved in the binding of Gβγ to RACK1. However, it should be pointed out that we cannot completely exclude the potential contribution of other regions of Gβγ in binding RACK1, because in this study we only evaluated the conserved domain between Gβγ and Gβδ, and it is possible that other regions also play a role in the Gβγ/RACK1 interaction. In addition, peptides with small effects on the Gβγ/RACK1 interaction that are beyond the sensitivity of our assays will not be detected. However, based on the following considerations, we believe that the region identified on Gβγ in this study is the key contact between Gβγ and RACK1. First, although the corresponding residues of the peptides p44–54, p86–98, p287–298, p309–316, and p328–337 are not contiguous in amino acid sequences, they are close in space and, particularly, clustered on a same surface of Gβγ and confined to three blades, blades 1, 6, and 7. This is consistent with the fact that, like Gβ, RACK1 is a member of WD40 repeat proteins that may form a rigid circular β propeller structure and thus its interaction with Gβγ may be mediated through limited contacts (13). Second, peptide p44–54 alone can inhibit up to 70% of the Gβγ/RACK1 interaction, and a combination of this peptide with p328–337 from the same blade 7 where p44–54 resides almost completely abolished the interaction, suggesting that residues 44–55 and 328–337 are the major molecular determinants of Gβγ for binding RACK1. In further support of this notion, mutations of 5 charged residues within amino acids 44–54 of Gβγ resulted in a weaker interaction between Gβγ γ2 and RACK1. Based on the crystal structure of the heterotrimeric Goαβγγ2, there are 16 residues from Gβγ engaged in interaction with the Go subunit (21–23). The peptides tested in this study contain 13 of these residues. However, only 3 Go-interacting residues, Lys-89, Ser-98, and Trp-332, are found to be within the RACK1 contact region of Gβγ (Fig. 5A). Because we could not clearly define the boundary of the RACK1 binding sites on Gβγ by using the peptide approach, it remains to be determined whether these residues are involved in direct interac-

FIGURE 10. Activation of PLCβ2 by wild-type and mutant Gβγ γ2. The activity of purified PLCβ2 in the presence of increasing concentrations of wild-type and mutant Gβγ γ2 was determined as described under “Experimental Procedures.” The EC50 and Fmax are 40 ± 7 nM and 100 ± 4% for wild-type Gβγ γ2, 7.7 ± 3 nM and 81 ± 12% for Gβγγ2 [R48/49A]γ2, 8.5 ± 4 nM and 65 ± 23% for Gβγγ2 [H54A]γ2, and 85 ± 12 nM and 54 ± 7% for Gβγγ2 [S5A]γ2.

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J. Biol. Chem. 2005, 280:33445-33452. doi: 10.1074/jbc.M505422200 originally published online July 28, 2005

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