A yeast two-hybrid approach was used to discern possible new effectors for the βγ subunit of heterotrimeric G proteins. Three of the clones isolated are structurally similar to Gβ, each exhibiting the WD40 repeat motif. Two of these proteins, the receptor for activated C kinase 1 (RACK1) and the dynein intermediate chain, co-immunoprecipitate with Gβγ using an anti-Gβ antibody. The third protein, AAH20044, has no known function; however, sequence analysis indicates that it is a WD40 repeat protein. Further investigation with RACK1 shows that it not only interacts with Gβγ but also unexpectedly with the transducin heterotrimer Gαβγ. Gα, alone does not interact, but it must contribute to the interaction because the apparent EC₅₀ value of RACK1 for Gαβγ is 3-fold greater than that for Gβγ (0.1 versus 0.3 μM). RACK1 is a scaffold that interacts with several proteins, among which are activated βIIPKC and dynamin-1 (1). βIIPKC and dynamin-1 compete with Gβγ and Gαβγ for interaction with RACK1. These findings have several implications: 1) that WD40 repeat proteins may interact with each other; 2) that Gβγ interacts differently with RACK1 than with its other known effectors; and/or 3) that the G protein-RACK1 complex may constitute a signaling scaffold important for intracellular responses.

Heterotrimeric G proteins are a family of proteins that transduce an extracellular signal to an intracellular response via a seven helical transmembrane G protein-coupled receptor (GPCR). Upon activation, the receptor facilitates the exchange of GDP for GTP in the Gα subunit. Gα is then thought to dissociate from the Gβγ heterodimer allowing both complexes to individually activate a number of effectors (2, 3). Free Gβγ interacts with a large assortment of effector proteins, including phospholipases (4), adenylyl cyclases (5), ion channels (6), and G protein-coupled receptor kinases (7). There are, however, G protein-coupled receptor responses, such as MAP kinase activation (8–10), receptor internalization (11, 12), and organelle transport (13–15) that are mediated through the Gβγ subunit but that have not been definitively linked to known Gβγ effectors.

Gβ is the prototypical member of a family of proteins known as WD40 repeat proteins, which seem to function as adaptors and enzyme regulators (16, 17). Gβ is the only WD40 repeat protein whose three-dimensional structure is known, and it exhibits a toroidal bladed β-propeller structure, with each blade consisting of 4 anti-parallel β-strands (18). Because the WD repeat motif is a structural element of the β-propeller, all of these proteins are thought to be β-propeller proteins with a variable number of blades. Furthermore, Gβ subunits are known to interact with Gγ subunits, proteins containing a Gγ-like domain (19), a pleckstrin homology domain (20), a QXXER domain (found in adenylyl cyclases) (21), and a domain contained within phosducin and its relatives (22). In this work we propose that Gβγ also interacts with certain other WD40 repeat proteins.

The dynein intermediate chain (DIC) is a WD40 repeat protein that is part of the cytoplasmic dynein multimeric protein complex, which consists of heavy, intermediate, light intermediate, and light chains (23). Dynein is a retrograde microtubule motor protein that is involved in cell division and in intracellular transport (24–26). The involvement of dynein in these processes is dictated by its different subunit composition (27) and by its interaction with another multimeric protein complex, dynactin (28–30). This latter interaction occurs through the intermediate chain of the dynein complex and through p150-glued of the dynactin complex (28, 31, 32). The regulation of the interaction between dynein and dynactin is not fully understood; however, phosphorylation plays a part (33–35).

The RACKs are also WD40 repeat proteins that were originally found by their ability to bind and to localize activated PKC (36, 37). Different RACKs interact with different PKC isoforms, and RACK1 interacts with βIIPKC (38, 39). More recently, RACK1 has been shown to interact with a variety of other proteins such as dynamin-1 (40), Src (41, 42), the β subunit of integrins (43, 44), p120GAP (45), PDE4D5 (46, 47), the interferon-α receptor (48), the β-chain of interleukin-5 receptor (49), and PTPζ (50) with some of these interactions being mutually exclusive and some being concurrent. RACK1 is now considered a scaffolding protein that allows specific multimeric complexes to form during different signaling events. Interestingly, four of these proteins, PKC (51–54), dynamin-1 (11, 55), Src (9, 10), and integrins (56), have also been linked to G protein signaling.
In this study, we used a yeast two-hybrid screen to find new Gβγ-interacting proteins and have isolated three proteins with the WD40 motif: RACK1, DIC, and an unknown protein, AAH20044. We confirmed the interaction of Gβγ with RACK1 and DIC through co-immunoprecipitation using an anti-Gγ1 antibody. Further investigation with a GST-RACK1 fusion protein and purified retinal G proteins revealed that RACK1 not only interacts with Gβγ1 alone but also binds with an apparent higher affinity to the heterotrimer Gαβγ1. Gαi alone does not bind to GST-RACK1, suggesting that the interaction is mainly through Gβγ. This result is surprising because very few proteins other than receptors are known to interact with the whole heterotrimer face (57, 58). Mutational analysis of RACK1 shows that G proteins may bind to multiple regions, including one of the βIPIPK-binding sites. Confirming this, Gβγ1 and Gαβγ1 compete with the binding of activated βIPIPK to RACK1. Finally, Gβγ1, RACK1, and dynamin-1 do not form a trimERIC complex, and dynamin-1 competes with the binding of Gβγ1 or Gαβγ1 to RACK1.

EXPERIMENTAL PROCEDURES

Materials—Recombinant dynamin-I baculovirus was a gift from Dr. Sandra Schmid (Scripps Institute), and pET-His-DIClA was a gift from Dr. Kevin Vaughan (University of Notre Dame). NIH3T3 cells were obtained from American Type Culture Collection. Transducin heterotrimer and individual a and βγ subunits were purified from bovine retinas as described (59).

Yeast Two-hybrid—The Hybrid Hunter Two-hybrid System (Invitrogen) and Matchmaker LexA Two-hybrid System (Clontech, PT3040-1) were employed. The cloning of cDNAs into vectors was done by standard molecular techniques. cDNA corresponding to bovine β1 was cloned into pLexA using EcoRI/XhoI sites, and the mouse brain library (Clontech) was cloned into pH42AD using EcoRI/XhoI sites. By using the EcoRI/XhoI sites, bovine γ2 was cloned into pHyLexA/Neo. The LexA binding domain of this plasmid was deleted beforehand by incorporating an EcoRI site upstream of the BD (Quick Change Mutation, Stratagene), referred to now as pHyb. The yeast strain EGY48/pSH18-34, which has a LEU2 site integrated in the genome and a lacZ plasmid, was employed. Transformation of the yeast was performed as outlined in the Matchmaker LexA Two-hybrid System User’s Manual. Interaction of the proteins was determined by the ability of the yeast to grow on leucine minus (leu−) media and the ability to turn blue in the presence of 5-bromo-4-chloro-3-indolyl β-galactosidase (X-gal). The “positive” filters were sequenced, and a BLASTN search was performed to determine the identity of the proteins (74).

Expression and Purification of Cytoplasmic Dynein Intermediate Chain-1A—A 10 ml LB/amp(100 μg/ml) overnight culture of Escherichia coli BL21 cells containing pET-DIClA was used to inoculate 500 ml of 2YT/amp. The culture was grown for 2.5 h (OD 0.6) at 37 °C and then induced with isopropyl-thio-galactoside (IPTG) (0.5 mM) for 3 h. Cells were collected and frozen. Cells were thawed at room temperature and resuspended in 15 ml of washing buffer (20 mM imidazole; 0.5 mM NaCl; 20 mM Tris-HCl (pH 7.9); protease inhibitor cocktail (PIC) (Amersham Biosciences); and 1% Triton X-100). Cells were sonicated on ice and then spun at 50,000 rpm for 1 h at 4 °C. Supernatants were collected and poured over a 2.0 ml amylose resin (New England Biolabs) column, which had been pre-washed twice with 20 ml of column buffer. Resin was washed four times with 20 ml of column buffer, then eluted with 4 ml of elution buffer (column buffer and 10 mM maltose), and then dialyzed into 40% glycerol/column buffer. MBP-RACK1 was >95% pure as determined by SDS-PAGE/Coomassie.

Expression and Purification of Dynamin-1—Expression and purification were carried out as described (60). Dynamin-1 was stored in GTB/ampase (50 mM Na-HEPES (pH 7.2), 10 mM MgSO₄, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween, 0.1 mg/ml bovine serum albumin). Dynamin-I was >95% pure.

Immunoprecipitation with Recombinant and Purified Proteins—Proteins were incubated at 4 °C with constant shaking for 30 min in a final volume of 200 μl using PBS for DIC/Gβγ and column buffer for MBP-RACK1/Gγ1. 1 μg of antibody (Santa Cruz) was added and incubated at 4 °C for 30 min, followed by the addition of 50 μl of a 50% slurry of protein A-Sepharose (Amersham Biosciences) and incubated at 4 °C for 30 min. The beads were washed three times with 500 μl of either PBS or 1% Igepal or column buffer + 0.1% Igepal, transferred to new tubes on the third wash, boiled in loading buffer, and resolved with SDS-PAGE (10–20%).

Immunoprecipitation with Endogenous Proteins—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, ampicillin (100 μg/ml), and streptomycin (100 μg/ml). When stated, cells were serum-starved for 6 h. A 100-ml plate of cells at ~85% confluence was lysed with 1.5 ml of RIPA buffer (100 mM Tris-HCl (pH 7.5), 1% Igepal, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor mixture) or with Triton buffer (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, and PIC) at 4 °C with constant rotation for 30 min. Plates were scraped, and lysates were collected and centrifuged at 14,000 rpm for 45 min at 4 °C. The supernatants were collected and incubated with 2 μg of anti-Gγ antibody overnight at 4 °C with constant shaking. The supernatants were incubated with 40 μl of a 50% slurry of protein A-Sepharose for 1 h at 4 °C with constant shaking. The supernatants were washed three times with buffer, transferred to new tubes on the third wash, boiled in loading buffer, and resolved with SDS-PAGE (10–20). The Triton-insoluble pellet was resuspended in 100 μl of PBS with 1% SDS and sonicated to disrupt it. 10 μl was added to each lane.

Western Blot—The gels were transferred to polyvinylidene difluoride membranes using a semi-dry apparatus. The membranes were blocked with a 5% milk, PBS, 1% Igepal buffer for 30 min at 4 °C, followed by an overnight incubation with the appropriate antibody (DICT-1A (1:1000) (gift from Prof. Kevin Vaughan, University of Notre Dame); RACK1 (1:5000) and dynamin-1 (1:2000) (Transduction Laboratories); Gβ (1:10,000) (Santa Cruz Biotechnology); Got (1:10,000) (gift from Prof. David Manning, University of Pennsylvania); βIPIKC (1:2000) (Santa Cruz Biotechnology)). The appropriate secondary antibody, conjugated to horseradish peroxidase, was incubated with the membrane for 1 h at room temperature. Detection was performed with chemiluminescence (Kirkgaard & Perry Laboratories or Pierce) by either x-ray film or a Fluor-S Imager (Bio-Rad).

GST-RACK1 Mutations—The mutations N1 (RACK1 amino acids 1–203), C1 (amino acids 203–317), N2 (amino acids 1–111), and C2 (amino acids 113–207) were created using standard molecular cloning techniques. For N1 and C2, we made use of an internal BamHI site of RACK1. The full-length RACK1 in pGEX-4T (Amersham Biosciences) was cut with BamHI (there is a BamHI site in the multiple cloning site of pGEX-4T, which gives a terminal fragment that was ligated into pGEX-4T and a pGEX-4-N-terminal RACK1 fragment that was ligated back together. For the N2 and C2 mutants, an EcoRI site was incorporated into pGEX-4T-N1-RACK1 using Quick Change site-directed mutagenesis, which changed alanine 112 to a glutamine. This construct was cut with EcoRI, which gives a C2 fragment and pGEX-
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4-N2-RACK1 fragment because of an EcoRI site in the multiple cloning site. The N2 fragment was ligated back onto itself, which subsequently incorporates an extra nine amino acids at the C terminus from the other restriction sites in the multiple cloning site. The C2 fragment was ligated into EcoRI-digested pGEX-4T. The correct clones were confirmed by sequencing.

**GST-RACK1 and Mutant Expression** — 10 ml of BL21 cells containing pGEX4T-RACK1 or one of the mutants were grown overnight in LB/amp (100 μg/ml) at 37 °C with shaking. 500 ml of LB/amp was inoculated with the overnight sample, grown at 37 °C for 4 h (A600 = 1–1.3), and then induced with 0.2 mM IPTG for 3 h. Cells were collected by centrifugation at 2000 rpm for 10 min at 4 °C and washed once with PBS, then resuspended in 5 ml of lysis buffer (10 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT).

**Fig. 1. Structure and sequence alignment of the WD repeat proteins.** A, ribbon representation of the Gβγ crystal structure (18) (PDB1TBG). Gγy, represented in blue, forms a coiled-coil interaction with the N terminus of Gβγ. The seven β-blades of Gγ, defined by a WD repeat, are highlighted in different colors. B, the sequences for Gγ (bovine) (CGG0B1), RACK1 (rat) (A36986), DIC-1A (mouse) (AAC33444), and the unknown protein (mouse) are shown. They were submitted to bmerc-www.bu.edu/bioinformatics/wdrepeat.html for analysis to predict WD repeat/β-blade formations. The sequences are aligned according to the WD repeat template (top of each blade alignment). Highlighted in blue are the amino acids important for hydrogen bonding and for stabilizing the toroidal structure, with the aspartic acid (italicized) being the most conserved residue (18). Arrows above the template represent which amino acids form the β-sheets, which in turn form each β-blade. The red arrow indicates the first β-sheet of the next blade. This is at the end of the variable loop region, which is in between each β-blade. DIC has only six WD repeats; therefore its WD7 is its C terminus.
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FIG. 2. Dynactin intermediate chain co-immunoprecipitates with Gβγ. A, in vitro immunoprecipitates (IP) of recombinant His-DIC (1 μM) and bovine Gβγ1 (1 μM). Lanes 1 and 2, IP using an anti-Gβ1 antibody (T20); lane 3, IP with no antibody. B, IP of endogenous DIC from NIH3T3 cells using an anti-Gβ antibody (T20). Lane 1, control, no antibody; lane 2, IP from cells in serum; lane 3, IP from serum-starved cells (no fetal bovine serum); lane 4, RIPA-soluble fraction from cells with serum; lane 5, RIPA-soluble fraction from serum-starved cells. All experiments were repeated three times, unless otherwise noted.

FIG. 3. RACK1 co-immunoprecipitates with Gβγ, A, in vitro immunoprecipitates (IP) of recombinant MBP-RACK1 (1 μM) and bovine Gβγ1 (1 μM). Lane 1, purified MBP-RACK1 (1 μg); lanes 2 and 3, IP using two anti-Gβ antibodies T20 (C terminus directed antibody) and M14 (N terminus directed antibody); lane 4, IP using an anti-Gγ antibody; lane 5, control, no antibody. B, IP of endogenous RACK1 from NIH3T3 cells using an anti-Gβ antibody (T20). Lane 1, purified MBP-RACK1 (0.5 μg); lane 2, control (no Gβγ); lane 3, IP from cells in serum; lane 4, IP from serum-starved cells; lanes 5 and 6, Triton-soluble and -insoluble fractions from cells with serum; lanes 7 and 8, Triton-soluble and -insoluble fractions from serum-starved cells.

PBS. The cells were frozen overnight at –80 °C. The cells were thawed on ice and resuspended in 15 ml of PBS, 1% Triton, 1 unit of PIC, sonicated, rotated for 1 h at 4 °C, and then centrifuged at 50,000 rpm for 1 h at 4 °C. Glycerol (50%) was added to the Triton-soluble fraction (supernatant), and the lysates were stored at –20 °C.

GST Binding Assays—0.5 ml of assay buffer (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 12 mM β-mercaptoethanol, and 1% polyethylene glycol) with 1 unit of PIC was added to the glycerol lysate volume to yield a final concentration of 300 mM GST-RACK in the 200-μl assay (protein concentration was determined by isolating all the GST-RACK1 or mutant from a 1-ml aliquot of the glycerol lysates and using a Bradford protein assay). Glutathione-Sepharose (Amersham Biosciences), 50 μl of a 50% slurry, was added to the diluted lysate. The mixture was incubated for 30 min at room temperature with rotation. The resin was washed three times with 500 μl of assay buffer. The appropriate amount of G protein was added, and assay buffer was added to a final volume of 200 μl. This was incubated for 30 min at room temperature, washed three times with assay buffer, transferred to new tubes on the third wash, boiled in loading buffer, and resolved with SDS-PAGE. Studies with βIIIPKC included an extra incubation for 30 min at room temperature with activated βIIIPKC before G protein was added. βIIIPKC was activated by incorporating phospholipid vesicles, consisting of dioleoylglycerol (2 μg/ml) and phosphatidylserine (60 μg/ml), to the assay, then βIIIPKC (50 nm), and then 1 mM CaCl₂ as described (39). Studies with dynamin-1 included an extra incubation of 30 min at room temperature after the addition of G protein.

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RESULTS

Yeast Two-hybrid Screen—Bovine Gβγ, fused to the C terminus of the LexA binding domain (BD), was used as bait in a yeast two-hybrid screen of a mouse brain library. The library consisted of open reading frames ~1 kb in length that were amplified from the poly(A) tail and fused to the C terminus of a yeast activation domain (AD). Prior to the screen, we determined that neither LexA-Gβγ, without the activating domain nor LexA-Gβγ2 with the activation domain alone gave a positive result in the assay.

Because Gβγ is normally associated with a Gγ subunit, it was important to determine whether LexA-Gβγ folds properly with endogenous yeast Gγ. When properly folded and exposed to trypsin, Gβ has only one exposed cleavage site (61). A tryptic digest of yeast lysates expressing LexA-Gβγ found that most of the LexA-Gβγ is folded properly, suggesting assembly with yeast Gγ; however, there was also some misfolded LexA-Gβγ1 protein (not shown). Therefore, we repeated the yeast two-hybrid assay with our positive clones in the presence of bovine Gγ2. Under these conditions, all of the LexA-Gβγ1 had only one tryptic cleavage site, indicating proper assembly with bovine Gγ2. Those clones that were positive when Gγ2 was present were further characterized.

To eliminate further false positives in the assay, we 1) confirmed the interaction of the isolated AD proteins with LexA-Gβγ; 2) determined if the isolated AD protein plus the BD alone was positive; and 3) determined if the AD protein alone gives a positive result. Unexpectedly, yeast with the AD protein and the LexA-BD alone grew on the leucine minus media; however, they did not turn blue, suggesting the promoter for LEU2 may be leaky. Table I outlines these results for the proteins presented here.

WD Repeat Protein—The three clones in Table I are all WD40 repeat proteins: RACK1, DIC, and an unknown protein (AD2–61). The unknown protein matches the sequence of a hypothetical protein with the GenBank™ accession number AAH200444 but has no known function. All of the proteins fell into the same family as Gβγ and are thought to exhibit the same β-propeller fold as Gβγ (Fig. 1A). When aligned according to their predicted WD motifs (Fig. 1B), the individual blades and the proteins show little sequence homology, except at key positions (highlighted in blue) as is usually seen for WD repeat proteins. Isolating three structurally similar proteins from a screen suggests the possibility that select WD repeat proteins may interact with each other.

Immunoprecipitations—We confirmed the interaction of Gβγ with RACK1 through co-immunoprecipitations (co-IP) (Fig. 2). A bacterially expressed human His-DIC-1A and bovine retinal Gβγ1γ2 are shown to interact in vitro (Fig. 2A). This interaction is further verified by showing that endogenous DIC co-immunoprecipitates with a Gβ antibody from NIH3T3 cells both in the presence and absence of serum (Fig. 2B, lanes 2 and 3, respectively).

We also confirmed the interaction of Gβγ with RACK1 through co-IPs (Fig. 3). A bacterially expressed rat RACK1 fused to the mouse-binding protein (MBP) and bovine retinal Gβγ1γ2 are shown to interact in vitro (Fig. 3A) using two different GST antibodies (lanes 2 and 3) and a Gγ1 antibody (lane 4). Again, the interaction is further verified by showing that endogenous RACK1 co-IPs with a Gβ1 antibody (T20) from NIH3T3 cells both in the presence and absence of serum (Fig. 3B, lanes 3 and 4). RACK1 Binds to Both Gβγ1γ2 and Gαβγ1γ2—Investigation using a GST-RACK1 fusion protein revealed that not only does Gβγ1γ2 interact with RACK1 but that heterotrimeric Gαβγ1γ2 interacts with it and with an apparent higher affinity (Fig. 4A, compare lanes 3 and 5 to 4 and 6). Equal amounts of GST-RACK1 were loaded in each lane (Fig. 4A, top blot), demonstrating that the difference in affinity is not due to unequal amounts of RACK1. Again, as shown in Fig. 4B, Gαβγ1γ2 has a higher affinity than Gβγ1γ2 for RACK1 (compare lanes 4 to 5); however, neither Gαγ1-GDP (lanes 6 and 7) nor Gαγ1-GTPγS (lane 8) interacts with RACK1. Furthermore, because Gαγ1 is present (middle blot, lane 4) in the pull-down assay, RACK1 must interact with the heterotrimer and not just displace Gαγ1 while binding to Gβγ1γ2. The interaction thus likely occurs through Gβγ1γ2; however, Gαγ1 must contribute directly or indirectly to the interaction, because the heterotrimer has a higher affinity.

Apparent EC₅₀ values of Gαβγ1γ2 and Gαβγ1γ2 for GST-RACK1 were determined by binding increasing amounts of each protein to a fixed amount of GST-RACK1 (Fig. 4C, blot). The amount of Gαβγ1γ2 or Gαβγ1γ2 pulled down was measured by densitometry and plotted as a percentage of maximum bound versus concentration (Fig. 4C, graph). As seen in the graph, 50% of Gαβγ1γ2 binds at a concentration of 0.1 μM whereas 50% of Gαβγ1γ2 binds at a concentration of 0.3 μM.

G Proteins Compete with Activated βIIPKC for Binding to RACK1—RACK1 was originally isolated by its ability to interact with activated βIIPKC (62). We wanted to determine whether Gαβγ1γ2 or Gαβγ1γ2 binds to RACK1 concurrently or competitively with activated βIIPKC. Fig. 5A demonstrates that increasing the concentration of Gβγ1γ2, either as heterotrimer or as heterotrimeric, decreases the amount of activated βIIPKC bound to GST-RACK1 and increases the amount of Gβγ1γ2 bound (compare lanes 1 to 2 for Gβγ1γ2 and lanes 3 and 4 for Gαβγ1γ2). Because there was a reduction of βIIPKC and an increase in Gβγ1γ2, the binding seemed to be competitive. However, higher concentrations of Gβγ1γ2 (1 μM) never completely inhibited the binding of βIIPKC to GST-RACK1 (not shown).

Because the interaction seemed to be competitive but not mutually exclusive, we wanted to narrow down the site on RACK1 that interacts with Gβγ. We constructed N-terminal (N1) and C-terminal (C1) mutants of RACK1 expressed as GST fusion proteins (Fig. 6A). Analysis of these two mutants (Fig. 6B) shows that G proteins interact with N1-RACK1 (lanes 6 and 7) and not with C1-RACK1 (lanes 8 and 9). To further define the interaction region on N1-RACK1, we divided this segment into two halves (Fig. 6A). However, this failed to narrow down one specific Gβγ-binding site, because both truncation mutants, N2-RACK1 and C2-RACK1, bind Gαβγ1γ2 and
Gβ1γ1 (Fig. 6B, lanes 10 and 11). This result suggests that Gβ1γ1 has multiple sites of interaction on RACK1, with probable overlap on one of the βIIIPKC-binding sites (63). This may explain why Gβ1γ1 competes with activated βIIIPKC but never fully inhibits its binding to RACK1.

Gαβ1γ1 and Gβ1γ1 Differ in Their Interaction with RACK1 When Dynamin-1 Is Present—A second RACK1 interacting protein is dynamin-1 (40), which also interacts with Gβγ (11, 55). We wanted to determine whether Gαβ1γ1 or Gβ1γ1 bind to RACK1 concurrently or competitively with dynamin-1. Keeping GST-RACK1 and Gαβ1γ1 or Gβ1γ1 constant, while increasing the concentration of dynamin-1 (Fig. 7), showed distinctively different results between Gαβ1γ1 (lanes 2–6) and Gβ1γ1 (lanes 7–11). For Gαβ1γ1, dynamin-1 excluded it from GST-RACK1 but not until its highest concentration (lane 6), demonstrating competition. For Gβ1γ1, the lowest concentration of dynamin-1 excluded it from GST-RACK1 (lane 8). Interestingly, very little dynamin-1 bound to GST-RACK1 at its lower concentrations (lanes 8 and 9); however, there is dynamin-1 bound at its higher concentrations (lanes 10 and 11). This also demonstrates competition, but the dynamics of the interaction are more complex because Gβ1γ1 also interacts with dynamin-1 (11, 55). A probable explanation for these results is that all of the proteins compete for binding to each other but that the affinity of Gβ1γ1 and dynamin-1 is higher than that of RACK1 for either protein. At the lower concentrations of dynamin-1, it was all bound to the Gβ1γ1, preventing it or Gβ1γ1 from binding to GST-RACK1. At the higher concentrations of dynamin-1, there was an excess of protein that was not bound to Gβ1γ1 and so in turn bound GST-RACK1.

DISCUSSION

WD Repeat Motif—A recurrent theme in signaling processes has emerged, that of multimeric protein complexes with activators and effectors bound together by adaptors in precise spatial arrangement to ensure proper cellular signaling. The association of multiple WD repeat proteins with Gβγ from a yeast two-hybrid screen suggests a new interaction motif. One could easily imagine WD repeat proteins as the scaffold for such a multimeric protein complex. Gβγ was the first WD repeat protein crystallized, and because the WD repeat is a structural element for the β-propeller, all WD repeat proteins are thought to exhibit the same toroidal shape as Gβγ (Fig. 1A) with variations on the number of repeats (16, 17). When trying to visualize the interaction of two WD repeat proteins with each other, it would be interesting to consider if it occurs top-to-bottom, side-to-side, side-to-top, or side-to-bottom. Structural studies of WD repeat protein complexes would help us understand the basis of their interaction.

Interaction between Gγ and DIC—The evidence presented for this interaction consists of a yeast two-hybrid interaction and co-IPs. Any discussion of its function must be based on the known literature. Furthermore, the interaction (Fig. 2) is weak when compared with the interaction of Gβγ with RACK1 (Fig. 3) and may not be absolute. However, there may be binding cofactors that facilitate the interaction, i.e. when DIC is in
complex with its light and heavy chains. This has preceded in the interaction of G by with the exocytotic machinery; G by has a higher affinity for the complex than the individual components (64).

Several interesting findings stand out in the literature that could lead to possible functional roles of the G by and DIC interaction. One is the finding that tctex-1, a dynein light chain, interacts with the C terminus of rhodopsin, a GPCR (27, 65), which is shown to be important for apical transport. The light chains of dynein interact directly with the intermediate chains (23), which raises the question: do G proteins help regulate apical transport through the interaction of G by with DIC? Another scenario could have G proteins helping to define the intracellular role of dynein by regulating its interaction with dynactin (28, 31, 32). This interaction is regulated by phosphorylation (34, 35); however, it has not been clearly defined (24). Could G proteins help provide specificity for the involvement of dynein in cell division and/or intracellular transport, cellular processes that also involve heterotrimeric G proteins (14, 15, 66–68)? The literature poses several other avenues that could be pursued, and it will be interesting to discern the functional role(s) of this interaction.

Interaction between G by and RACK1—At first, our interest in this interaction was initiated by a possible connection to undefined G by-mediated responses. RACK1 interacts with both activated βIPKC (37) and dynamin-1 (40), two proteins that are also linked to heterotrimeric G proteins. βIPKC is a serine/threonine kinase activated downstream of phospholipase C (69) and subsequently heterotrimeric G proteins (70). PKCs are important second messengers (71) with many substrates, among which are the Gs subunits i, z, 12, and 16 (51–54). Phosphorylation of the N terminus of Gs prevents its re-association with the G by subunit, possibly prolonging signaling through both subunits. Dynamin-1 is a large GTPase involved in clathrin-coated receptor endocytosis (72) and more recently has been linked to the MAP kinase pathway (73). Interestingly, RACK1 and G by have opposite effects on dynamin-1: G by inhibits its GTPase activity (55), and RACK1 increases it (40). These literature findings suggest that there may be a dynamic interaction between these proteins leading to an intracellular response such as signal attenuation, MAP kinase activation, and/or receptor endocytosis.

The interaction of RACK1 with G by was confirmed through co-IPs (Fig. 3). Subsequent studies using GST-RACK1 suggests that it not only interacts with G by, a single α subunit, but also in the heterotrimeric form, Gαβγγ, with an apparent higher affinity (Fig. 4). This was very surprising for the following two reasons: 1) very few proteins, other than receptors, interact with the whole heterotrimer (2); and 2) all known G by effectors bind to its Go-interacting face (57, 58). RACK1 does not interact with Gα alone; however, the Gs subunit must contribute to the interaction because the heterotrimer has a 3-fold higher affinity than the heterodimer for RACK1 (compare EC50-Fig. 4C). Gα could stabilize a conformation of G byγ that binds better to RACK1 or Gα could participate in the interaction but binds with a higher affinity than it can bind RACK1 alone. Structural studies are needed to determine the involvement of Gα in the interaction.

To discern a function of this interaction, we first explored the involvement of activated βIPKC (Fig. 5). Both Gβγ and Gαβγγ compete with the binding of activated βIPKC to GST-RACK1 but do not entirely block the interaction. Mutational analysis of RACK1 (Fig. 6) suggests that Gβγγ has multiple RACK1-binding sites, with probable overlap on one of the βIPKC-binding sites. This could cause competition with βIPKC binding to RACK1 but not full exclusion.

Extending our studies to dynamin-1 (Fig. 7), we found that it competes with the binding of Gαβγγ to RACK1 but not until its highest concentration (1 µM). There is also a competition between dynamin-1, RACK1, and Gβγγ; however, it is more dynamic because all three proteins can interact with each other. Dynamin-1 competes with the binding of RACK1 to Gβγγ in a way that suggests the affinity of Gβγγ and dynamin-1 is higher than that of RACK1 and dynamin-1 or that of RACK1 and Gβγγ.

Implications for G Protein Signaling—The importance of the interaction of G by with RACK1 still remains to be determined; however, one interesting scenario concerns the localization of RACK1 to the membrane. RACK1 is found associated with the plasma membrane (1), yet it is not known to be post-translationally modified in any way that would promote membrane association, and it is not known to interact with a Gx-like domain-containing protein to localize it to the membrane, such as Gβ (19). Interestingly, Somodek and Siderovsk (19) propose that RACK1 interacts with PDE4D5 through a Gx-like domain. Could the interaction of RACK1 with G by be its mechanism of recruitment and association with the membrane, reminiscent of Gβγ and GRK2 (7)? One could imagine a Gβγγ-localized, membrane-associated RACK1, which after stimulation of a Gαγγ-coupled receptor and subsequent activation of PKC, would be in a position to help recruit activated PKC to the membrane (36). PKC would then be able to phosphorylate its desired substrates at the membrane, which include various Gα subunits (51–54).

Another approach to elucidate a role for this interaction is to determine the effect RACK1 has on heterotrimeric G proteins. The surprising result that RACK1 interacts with the Gαβγγ heterotrimer as well as with the Gβγγ heterodimer raises several questions. First, is the interaction of RACK1 with heterotrimeric G proteins subunit-specific? Second, is the increase in affinity of RACK1 for heterotrimer over heterodimer seen further studies will verify these interactions and quantify their affinities for RACK1.

There still remain other questions that can be asked for defining the functionality of this interaction. What is the importance of the interplay with dynamin-1? Is there a connection to Src or β integrin signaling in that both Gα proteins (9, 10, 56) and RACK1 (41–44) are involved in the regulation of Src-mediated and integrin-mediated signaling? The interaction between these two proteins has initiated more questions than it has resolved. Nonetheless, these initial studies have provided us with a solid foundation that could lead us to further insight into the dynamics of cellular signaling.

REFERENCES
1. Schectman, D., and Moehly-Rosen, D. (2001) Oncogene 20, 6339–6347
2. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
3. Neer, E. J. (1995) Cell 80, 249–257
4. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
5. Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 461–480
6. Schneider, T., Igelmann, P., and Heschler, J. (1997) Trends Pharmacol. Sci. 18, 8–11
7. Fisher, J. A., Inglese, J., Higgin, J. B., Ariza, J. L., Casey, P. J., Kim, C., Benovin, J. L., Kwastra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
8. Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12706–12710
9. Luttrell, L. M., della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 4637–4644
10. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and

a S. Chen, E. J. Della, and H. E. Hamm, unpublished data.
