By means of a variety of intracellular scaffolding proteins, a vast number of heterotrimeric G protein–coupled receptors (GPCRs) may achieve specificity in signaling through a much smaller number of heterotrimeric G proteins. Members of the tetraspanin family organize extensive complexes of cell surface proteins and thus have the potential to act as GPCR scaffolds; however, tetraspanin-GPCR complexes had not previously been described. We now show that a GPCR, GPR56/TM7XN1, and heterotrimeric G protein subunits, Gaq, Gai1, and Gβ3, associate specifically with tetraspanins CD9 and CD81, but not with other tetraspanins. Complexes of GPR56 with CD9 and CD81 remained intact when fully solubilized and were resistant to cholesterol depletion. Hence they do not depend on detergent-insoluble, raft-like membrane microdomains for stability. A central role for CD81 in promoting or stabilizing a GPR56-CD81-Gaq11 complex was revealed by CD81 immunodepletion and reexpression experiments. Finally, antibody engagement of cell surface CD81 or cell activation with phorbol ester revealed two distinct mechanisms by which GPR56-CD81-Gaq11 complexes can be dynamically regulated. These data reveal a potential role for tetraspanins CD9 and CD81 as GPCR scaffolding proteins.

**INTRODUCTION**

Heterotrimeric G-protein–coupled receptors (GPCRs) are the largest family of cell surface receptors, accounting for >1% of the human genome. GPCRs transduce extracellular signals from odorants, tastants, photons, small molecule and peptide hormones, growth factors, morphogens, and neurotransmitters (Bockaert and Pin, 1999; Marinissen and Gutkind, 2001; Pierce et al., 2002). The critical physiological roles of GPCRs have been repeatedly confirmed in mouse knockout models (Rohrer and Kobilka, 1998) and studies of human heritable diseases (Stadel et al., 1997). GPCRs are also among the most frequent targets of therapeutic drugs (George et al., 2002). In the classical GPCR signal transduction paradigm, ligand binding induces a conformational change in the GPCR that is transmitted to an associated, cytoplasmic heterotrimeric G protein. GDP bound to the G protein Ga subunit dissociates and is replaced with GTP, triggering the dissociation of the Ga subunit from the Gβγ subunits. The dissociated subunits bind and activate downstream effectors until the GTP bound to the Ga subunit is hydrolyzed to GDP, promoting the reassociation of Ga and Gβγ subunits (Pierce et al., 2002).

A major challenge in the study of GPCRs has been to explain how the ∼1000 GPCRs in the human genome are able to achieve specificity in signaling through ∼20 heterotrimeric G proteins. Recent advances highlight the importance of intracellular scaffolding proteins, including PDZ domain, SH2 domain, and polyproline-binding proteins, in organizing GPCRs into GPCR-specific signaling complexes (Hall et al., 1999; Pierce et al., 2002). Scaffolding could add to specificity by linking subsets of GPCRs to specific cytoplasmic signaling proteins. Alternatively, molecular scaffolds might segregate GPCRs in proximity with specific downstream targets.

Members of the tetraspanin family of cell surface proteins may act as molecular scaffolds by forming complexes with other cell surface proteins, including integrins, IgSF proteins, proteoglycans, growth factor receptors, membrane-bound growth factors, and other tetraspanins (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2003). The large number of tetraspanin-associated proteins is envisioned as being organized into a “tetraspanin web” or a network of tetraspanin-enriched microdomains (TEMs). However, despite the wide variety of proteins reported to be in TEMs, GPCR-tetraspanin associations have not yet been reported.

We applied a sensitive mass spectrometry protein sequencing approach to search further for proteins uniquely present in complexes organized by tetraspanins, CD9 and CD81. We previously showed that CD9/CD81 complexes isolated from Brij 96 detergent lysates have a size range significantly <4 million daltons, are fully solubile, and are resistant to cholesterol depletion with methyl-β-cyclodextrin (Stipp et al., 2001b). Our characterization of these CD9/CD81 complexes (Stipp et al., 2001a, 2001b), together with two other independent studies (Charrin et al., 2001; Clark et al., 2001), identified two major proteins within the complexes as EWI-2/PGL and EWI-F/CD9-P1/FPRP. These novel IgSF protein subfamily members associated specifically with CD9 and CD81 under conditions where associations with other tetraspanins were not detected (Charrin et al., 2001; Stipp et
al., 2001a, 2001b). Thus complexes organized by CD9 and CD81 are distinct from other tetraspan complexes.

We now report that an orphan heterotrimeric G protein-coupled receptor, GPR56, and heterotrimeric G protein subunits, Gαq/11 and Gβ, also associate specifically with tetraspanins CD9 and CD81. Furthermore, we demonstrate that CD9 plays a central role in CD81-GPR56-Gq/11-Gβ complexes. Finally, the GPR56-CD81-Gq/11-Gβ complex is dynamically regulated on intact cells, as anti-CD81 antibody triggered dissociation of Gq/11 and Gβ from GPR56-CD81-L, whereas phorbol ester induced dissociation and sequestration of GPR56 from CD81-Gq/11-Gβ. Our results are consistent with a role for CD9 and CD81 as scaffolding proteins in GPCR signal transduction.

MATERIALS AND METHODS

Antibodies

Anti-tetraspan mononclonal antibodies were used anti-CD81, M8 (Fukuda et al., 1992) and 564 (Pisano et al., 1986); anti-CD9, ALB6 (Chemicon, Temecula, CA); anti-CD63, 6H1 (Berditchevski et al., 1995); and anti-CD131, SC1 (You et al., 1998). M2 anti-FLAG epitope antibody, in free form, or conjugated to biotin or agarose, was purchased from Sigma (St. Louis, MO). Anti-heterotrimeric G protein subunits were used anti-αq, AE-9E (Lee et al., 1995), anti-α2, A2-IE10 (Bergerelson et al., 1994), and anti-β1, TS2/16 (Hemler et al., 1984). Other monoclonal antibodies were anti-CD147, 8G6 (Berditchevski et al., 1995), anti-CD9, Vim36 (Research Diagnostics, Inc., Flanders, NJ) and anti-CD81 antibodies used were rabbit anti-Gαq/11 (sc-263, sc-262), anti-Gq/11 (sc-262), and anti-Gβ (sc-261), all from Santa Cruz Biotechnology (Santa Cruz, CA), horseradish peroxidase-(HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies (Transduction Labs, Lexington, KY or Sigma), and fluorescein isothiocyanate–conjugated goat anti-mouse antibodies (Biosource International, Camarillo, CA).

Cell Culture

Human embryonic kidney cells (HEK293), HT1080 fibrosarcoma cells, NT2 embryonic carcinoma cells, and PT67 retroviral packaging cells (CLONTECH, Palo Alto, CA) were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Life Technologies), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Retinoid acid-differentiated NT2 cells were obtained by treating NT2 cells with 10 μM retinoic acid for 5 days, splitting 1.6 into fresh flask, and treating for 14 days with mitotic inhibitors (Pleasure et al., 1992). U937.C2, a subclone of U937 monocyctic lymphoma cells that do not express CD81, were generously supplied by Shoshona Levy, Stanford University Medical Center. These cells were cultured in RPMI media (Life Technologies) supplemented with 10% fetal bovine serum, glutamine, and antibiotics, as described above.

Purification of CD81 Complexes and Tandem Mass Spectrometry and Sequencing

CD81-associated proteins were prepared as previously described (Stipp et al., 2001b). Briefly, CD81 complexes were affinity-purified from a B96 lysate of NT2RA cells using anti-CD81 mAb 364 and protein G-Sepharose. CD81-associated proteins were eluted in 1% Triton, resolved by SDS-PAGE, and silver-stained. Individual gel regions were excised, rinsed with 50% HPLC grade methanol, and digested by complete DNA sequencing. MS/MS spectra of the peptides was facilitated by programs developed in the Harvard Microchemistry Facility and by database correlation with the algorithm Sequest (Eng et al., 1994; Chittum et al., 1998).

Construction of GPR56, CD97, and CD81 Expression Vectors

To construct epitope-tagged human GPR56 (also known as TMTXN1) expression vectors, we first obtained a GPR56 cDNA (GenBank accession number A610263.1, IMAGE clone ID 2187776) in the vector pCMV-SPORT 6.0 from Research Genetics Inc. (Birmingham, AL) using PCR, this cDNA was repaired at the end of the coding region so that it exactly matched a published GPR56 sequence (Zendman et al., 1999), as verified by complete DNA sequencing. Using the repaired GPR56 cDNA as a template, additional rounds of recombinant PCR with Pfu polymerase were performed to 1) insert a FLAG epitope tag between Glu30 and Asp31, downstream of the putative signal peptide cleavage site at Gly32; 2) in a separate construct, add a FLAG epitope tag to the carboxy terminus of GPR56, after the final residue, Leu319; 3) add a Sull restriction site at the 5′ end of the coding region of each epitope-tagged construct; and 4) add a Nol restriction site at the 3′ end of the coding region of each construct. The resulting amino-terminally tagged (NFLGPR56) and carboxy-terminally tagged (CFLGPR56) cDNAs were cloned into the Sull and Nol sites of the pcLXZ retroviral vector. Final constructs were verified by sequencing.

A full-length human CD97 cDNA (isoform 2) in pCMV-SPORT 6.0 (GenBank accession number BC0010115; IMAGE clone number 9068; Research Genetics Inc.) was obtained and sequenced in its entirety. The CD97 cDNA was then excised from pCMV-SPORT 6.0 with Sull and Nol restriction enzymes and cloned into the pcLXZ retroviral vector, as for GPR56 constructs above. Human CD81 cDNA (GenBank accession number BE762318; IMAGE ID number 3872952) was obtained from Research Genetics, Inc. After verifying the cDNA by sequencing, Xhol and EcoRI restriction sites were added to the coding 5′ and 3′ ends, respectively, using Pfu polymerase in PCR. The resulting cDNA was cloned into the Xhol and EcoRI restriction sites of the pLXIN retroviral vector and reverified by sequencing.

Retroviral Transduction

Retroviral expression constructs described above were transfected into PT67 packaging cells using the Superfect reagent (QIAGEN, Chatsworth, CA). Forty-eight hours after transfection, PT67 cell supernatants were passed through a 0.45-μm filter, supplemented with 4 μg/ml polybrene (Sigma), and used to infect naïve PT67 packaging cells that had been pre-treated with 200 ng/ml tunicamycin (Sigma) for 18 h. Stable virus-producing cells were obtained by selection for 2 weeks in 0.5 mg/ml zeocin (Invitrogen, Carlsbad, CA) or G418 (Gibco BRL, Rockville, MD) as appropriate. The supernatant from these PT67 cell cultures was harvested, filtered, and supplemented with polybrene as above and then was used to infect target 293 or U937 cells. Transfected cells were selected for 2 weeks in 0.5 mg/ml zeocin or 0.5 mg/ml G418 and then maintained in 0.1 mg/ml selectin throughout. U937 cells were maintained in bulk, uncloned populations. For U937 cell lines, immunoselection with magnetic anti-CD81 or anti-FLAG beads was used to separate CD81 and NFLGPR56-expressing cells (~50% of the uncloned population) from a population of nonexpressing cells.

Immunoprecipitation and Immunoblotting

Cells were lysed in 1% detergent—Brij 96, (Fluka, Ronkonkoma, NY), or Brij 99, CHAPS, or Triton X-100 (all supplied by Sigma)—in 20 mM HEPES, pH 7.3, 150 mM NaCl, and 5 mM MgCl2 (HBSM) with 2 mM phenylmethylsulfonyl fluoride (Sigma), 20 μg/ml aprotinin, and 10 μg/ml leupeptin (Roche Molecular Biochemicals, Indianapolis, IN). In some experiments, cells were surface labeled with 0.2 mg/ml sulfo-NHS-LC biotin (Pierce, Rockford, IL) in HBSM for 1 h at room temperature and rinsed thoroughly in phosphate-buffered saline (PBS) before lysis. Cells were extracted on a rocker at 4°C for 1–3 h, and insoluble material was removed by centrifugation at 15,000 × g for 15 min at 4°C. Supernatants were preclarified for ±1 h with protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), or, for M2 anti-FLAG agarose immunoprecipitations, with mouse IgG (Sigma) conjugated to agarose beads (Affigel-10; Bio-Rad, Hercules, CA), then centrifuged as above. Surface monoclonal antibodies were added along with protein G-Sepharose, and immune complexes were collected overnight at 4°C with rocking. After four rinses with respective lysis buffer, samples were boiled in Laemmli buffer, resolved by nonreduced SDS-PAGE, and transferred to a nitrocellulose membrane.

For biotinylated samples, blots were blocked with 3% nonfat dry milk (w/v) in PBS with 0.01% Tween 20 (PBST, Fisher, Pittsburg, PA) for 1 h at room temperature and rinsed thoroughly with PBST. Blots were developed with a 1-h exposure to HRP-conjugated Extravidin (Sigma) followed by four rinses with PBST and chemiluminescence detection (Western Lightning reagent; Perkin-Elmer Cetus, Boston, MA). For immunoblotting filters were blocked in 5% nonfat milk in PBS for 1 h. The filters were then rinsed twice and treated and treated for 1–2 h with primary antibody diluted in blocking buffer. After four rinses with PBST, blots were developed with a 1-h exposure to HRP-conjugated secondary antibody followed by chemiluminescence as above. Fluorescent immunoblots were developed with biotinylated M2 anti-FLAG antibody according to the manufacturer’s directions. Semi-quantitative densitometry was performed using GeneTools software (Syngene, Frederick, MD) on digitized images captured from trans-illuminated films with a CCD camera driven by GeneSnap software (Syngene).

Equilibrium Density Gradient Centrifugation

Cell surface biotinylated NFLGPR56-expressing 293 cells were lysed in 1% Brij 96 as above. Lysate (1 ml) containing ~9 × 108 cell equivalents was mixed with 1 ml of 90% sucrose, 1% Brij 96 in HBSM, loaded over a 0.5-M cushion of 50% sucrose, and overlaid with layers of 40% sucrose (1 ml), 20% sucrose (1 ml), and 5% sucrose (0.5 ml) prepared in HBSM without detergent. After centrifugation for 21 h at 45,000 rpm in a Beckman SW28 rotor (Fullerton, CA) at 4°C, fractions were collected from the top of the gradient. One milliliter
Table 1. Peptides recovered from CD81-associated proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptides</th>
<th>Est cluster; GenBank accession no.</th>
<th>Gel region</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM7XN1/GPR56</td>
<td>1. (R) RPSAAPASQQLSLESK</td>
<td>AJ011001</td>
<td>70–90 kDa</td>
</tr>
<tr>
<td></td>
<td>2. (R) DLQLLSQFLK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. (R) LQPTAGLQDLIHISR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goq/11</td>
<td>4. (K) ILYSHLVDFEPFDFQQR</td>
<td>AF011497</td>
<td>40–60 kDa</td>
</tr>
<tr>
<td></td>
<td>5. (R) IATLYGPLTQDVLR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goq/6</td>
<td>6. (R) VADPAYLPTQDVLR</td>
<td>U43083</td>
<td></td>
</tr>
<tr>
<td>gβ/11</td>
<td>7. (R) LLVSAQSGDK</td>
<td>Multiple (see text)</td>
<td>20–40 kDa</td>
</tr>
</tbody>
</table>

Aside from those peptides listed, no other peptides were obtained for GPCRs, heterotrimeric G proteins, or any of their previously described associated proteins.

of 1.36% Brij 96 in HB8M was added to each fraction, and NFLGPR56 complexes were immunoprecipitated with anti-FLAG agarose and analyzed by blotting with Extravidin-HRP, as described above.

**Immunodepletion**

Approximately 1.8 × 10^7 NFLGPR56-expressing 293 cells were surface-labeled with biotin, rinsed, and lysed in 1% Brij 96, as described above. The lysate was divided into three parts and depleted three times for 1 h with a) protein G-Sepharose alone (mock), b) TS2/16 anti-β1 integrin agarose, and c) M38 anti-CD81 agarose. Depleted samples were further divided into four parts: three parts of 10% each for immunoprecipitation with anti-CD81, FLAG, or β1 integrin antibody followed by detection with Extravidin-HRP, and one part of 70% for immunoprecipitation with FLAG antibody followed by immunoblotting for Goq/11.

**Anti-CD81 Triggering Experiments**

Intact cells or membrane fraction (see below) were exposed to anti-CD81 antibodies, M38 or J64, or to anti-CD151 antibody, SC11 for 5–15 min at 37 or 0 °C. Cells were then rinsed and lysed in 1% Brij 96 as described above; membranes, in 100 µl, were extracted with the addition of 900 µl of 1.1% Brij 96. After a 1-h extraction, insoluble material was removed by centrifugation, and lysates were precleared with CL-6B-Sepharose or with agarose-conjugated mouse IgG; no protein G-Sepharose was used during the preclearing steps of these experiments because specific antibodies had been introduced before lysis. In one experiment, M38 anti-CD81 antibody was added to the lysate during the preclarifying step as a control for unintended immunodepletion. After preclarification, samples were immunoprecipitated and analyzed by immunoblotting for Goq/11, Gβ, CD81, or FLAG epitope as described above.

**Membrane Preparation**

NFLGPR56-expressing 293 cells, 3.6 × 10^7, were scraped into 5.5 ml of cold homogenization buffer (20 mM HEPES, pH 7.3, 150 mM NaCl, and 5 mM MgCl2), with 2 mM PMSE, 20 µg/ml aprotinin, and 10 µg/ml leupeptin. Cells were disrupted by 10 strokes in a dounce homogenizer. The homogenate was centrifuged at 500 × g for 5 min at 4 °C, and the resulting supernatant was then further centrifuged at 100,000 × g for 40 min in an SW55 rotor at 4 °C to pellet the membrane fraction. The membranes were resuspended in 500 µl of homogenization buffer; 100 µl of this membrane suspension was either treated with M38 anti-CD81 or left untreated in the antibody triggering experiments described above.

**PMA Stimulation**

U937 cells were treated with the phorbol ester, 12-tetradecanoylphorbol 13-acetate (PMA), at 200 nM for 20 min at 37 °C. Cells were then rinsed and resuspended in normal growth medium at 37 °C for periods up to 2 h. At set intervals, cells were removed, rinsed in ice-cold PBS, and held on ice in 5% goat serum in PBS with 0.01% azide. After the final time point was collected, the cells were stained with M2 anti-FLAG or M38 anti-CD81 monoclonal antibodies, followed by FITC-conjugated goat anti-mouse secondary antibody, and analyzed by flow cytometry. In parallel experiments, PMA-treated cells were extracted with Brij 99 detergent before immunoprecipitation and immunoblotting, as described above.

**RESULTS**

**GPR56 Associates with CD9 and CD81**

To search for additional CD81-associated proteins, we used a previously described mass spectrometry protein sequencing approach (Stipp et al., 2001b). Fully solubilized CD81 complexes were affinity purified from a 1% Brij 96 detergent lysate of retinoic-acid–differentiated NT2 teratocarcinoma cells (see MATERIALS AND METHODS). CD81-associated proteins were separated by SDS-PAGE, silver-stained, excised, digested in situ with trypsin, and subjected to tandem mass spectrometry peptide sequencing. As shown in Table 1, we obtained three peptides (from the 70–90-kDa region of the gel) that corresponded to an orphan GPCR named GPR56 (also called TM7XN1 and, in mouse, Cyt28; Liu et al., 1999; Zemandman et al., 1999; Terskikh et al., 2001).

GPR56 is a member of a GPCR subfamily termed LN-TM7 (Zemandman et al., 1999) or LNB-TM7 (Stacey et al., 2000). This subfamily is characterized by transmembrane domain homology to the B clan of GPCRs and by unusually large N-termini (LN denotes “large N-terminus”) that can contain modules from other protein families, including EGF, immunoglobulin, cadherin, lectin, laminin, and thrombospondin-like domains (Stacey et al., 2000; Krasnoperov et al., 2002; Fredriksson et al., 2003). The GPR56 N-terminus contains none of these modules but does contain a GPCR proteolytic site (GPS), a putative cleavage site that is characteristic of the LNB-TM7 subfamily and is defined by a conserved pattern of cysteine residues (Krasnoperov et al., 2002). The GPR56 mRNA is present in certain poorly metastatic tumor cell lines (Zemandman et al., 1999), the thyroid gland, adult and developing rodent brain (Liu et al., 1999), and hematopoietic stem cells and neuronal precursors (Terskikh et al., 2001), but the GPR56 protein had not been studied.

To verify that GPR56 is a CD81-associated protein, we transduced two different GPR56 CDNAs into the human embryonic kidney 293 cell line to obtain 293-NFLGPR56 cells with an N-terminal FLAG epitope tag, and 293-CFLGPR56, with a C-terminal FLAG tag. Using anti-FLAG antibody, 293-NFLGPR56 cells were positive by flow cytometry (Figure 1A), whereas CFLGPR56 cells were indistinguishable from 293 IZ control cells, which had been infected with an empty retroviral vector. These results are consistent with an extracellular N-terminus and an intracellular C-terminus, as predicted for a GPCR.

Anti-FLAG epitope immunoprecipitations from 1% Brij 96 lysates revealed comparable levels of N- and C-terminally FLAG-tagged GPR56 proteins on the surface of 293 cells, and comparable association with CD9 and CD81-like proteins (Figure 1B, lanes 1 and 6). CD9 and CD81 also strongly associate with each other, as previously observed (Stipp et al., 2001a, 2001b). The identities of the individual CD9 and CD81 bands have been repeatedly established by immunoprecipitation in stringent buffers that disrupt CD9-CD81 interactions.
interactions (cf. Berditchevski et al., 1996). In contrast, no CD9- or CD81-like bands coprecipitated with the highly expressed integrin α2 or α6 subunits in 293 cells (lanes 2 and 5), as expected from previous results (Stipp et al., 2001a, 2001b). Within the CD9 and CD81 immunoprecipitates (lanes 3 and 4), surface-labeled 78-kDa GPR56 was not obvious. Instead, there appears to be abundant EWI-2/PGRL, a 70-kDa IgSF protein and major CD9 and CD81 partner (Clark et al., 2001; Stipp et al., 2001a). Lighter exposures (lanes 3’, 4’, and 6’) more clearly show that labeled proteins associated with CD9 and CD81 are indeed slightly smaller than GPR56. For comparison, we also expressed CD97, an-

Figure 1. Specific association of CD9 and CD81 with GPR56. (A) Nonpermeabilized 293-NFLGPR56 cells (solid black line), 293-CFLGPR56 cells (solid gray line), or 293 IZ empty vector control cells (broken black line) were stained with M2 anti-FLAG antibody followed by FITC-goat anti-mouse 2° antibody and analyzed by flow cytometry. (B) The indicated proteins were immunoprecipitated from a Brij 96 lysate of biotinylated 293-NFLGPR56 cells (lanes 1–5 and 8), 293-CFLGPR56 cells (lane 6), or 293-CD97 cells (lane 7). Dashes between lanes 7 and 8 designate CD97 and GPR56, respectively. For lanes 3, 4, and 6, lighter exposures are shown (lanes 3’, 4’ and 6’) to allow better resolution of the 70–80-kDa region. (C) The indicated proteins were immunoprecipitated from a 1% Brij 96 detergent extract of 293-NFLGPR56 cells, followed by immunoblotting with the M38 anti-CD81 mAb. Cell equivalents, 4.5 × 10⁶, were used for the immunoprecipitation in lane 1, and 4.3 × 10⁶ cell equivalents were used in lanes 2–4. Analysis of the blot by densitometry indicated that ~2% of total CD81 may be GPR56-associated. (D) Because of the limited sensitivity of the FLAG antibody in immunoblotting, coprecipitation of GPR56 with CD81 was analyzed by reimmunoprecipitation. About 1.8 × 10⁷ biotinylated 293-NFLGPR56 cells were lysed in 1% Brij 96 and GPR56 was immunoprecipitated directly from 5% of the lysate (lane 1). The remainder of the lysate was divided equally between CD151 and CD81 immunoprecipitations (lanes 2 and 3). Next, CD151- and CD81-associated proteins were eluted with Triton X-100 from protein G–bound CD151 and CD81 immune complexes and reimmunoprecipitated for FLAG-GPR56. Analysis by densitometry indicated that ~2% of total GPR56 may be CD81-associated. In A through D, antibodies used were M2 anti-FLAG agarose (for GPR56), A2-IIE10 anti-α2 integrin, Alb-6 anti-CD9, M38 anti-CD81, A6-ELE anti-α6 integrin, Vim3B anti-CD97, and 5C11 anti-CD151. Material in GPR56 lanes that is >150 kDa varied between experiments and may be SDS-resistant oligomers of GPR56.
other LNB-TM7 GPCR subfamily member, in 293 cells (293-CD97 cells). In the same experiment, comparable levels of cell surface biotin-labeled CD97 and GPR56 were recovered by immunoprecipitation (Figure 1B, lanes 7 and 8). However, only GPR56 coprecipitated CD9- and CD81-like bands (lane 9). Hence, not all LNB-TM7-subtype GPCR proteins associate with CD9 and CD81.

Confirming the identity of CD81 in GPR56 immunoprecipitations, CD81 was detected by immunoblotting both in the CD81-positive control immunoprecipitate (Figure 1C, lane 1) and the GPR56 immunoprecipitate (lane 3), but not in CD151 or α6 integrin immunoprecipitates (lanes 2 and 4). To complement results in Figure 1, B and C, a reciprocal experiment was carried out (Figure 1D). GPR56, CD151, or CD81 were immunoprecipitated from a Brij 96 extract of biotinylated 293-NFLGPR56 cells. We then eluted tetraspanin-associated proteins from bead-bound CD81 and CD151 using Triton X-100, reimmunoprecipitated with anti-FLAG agarose, and recovered GPR56 from the CD81 (lane 3), but not from the CD151 (lane 2) immunoprecipitation.

Biochemical Characterization of GPR56-CD9/CD81 Complexes

The amount of CD9 and CD81 that coprecipitated with GPR56 from biotinylated 293-NFLGPR56 cells was no greater in a mild CHAPS detergent extract (Figure 2A, lane 1) than in Brij 96 extracts (lanes 3 and 4), although a large number of other cell-surface biotinylated species were observed in CHAPS. This is consistent with a previous report in which tetraspanin complexes solubilized in CHAPS were quite large (in excess of 20 million daltons; Skubitz et al., 2000). In a Brij 99 extract, somewhat more CD9 and CD81 coprecipitated with GPR56 than in either CHAPS or Brij 96 extracts (Figure 2A, lane 2), but far fewer additional species coprecipitated than in CHAPS. This is consistent with the intermediate stringency of Brij 99 for preserving tetraspanin interactions and the intermediate size of Brij 99-solubilized complexes (Claas et al., 2001). A significant fraction (~50–60%) of the GPR56-associated CD9 and CD81 observed in Brij 99 was preserved in Brij 96 lysates (lanes 3 and 4), regardless of whether the complex was captured in a 3- or 18-h overnight immunoprecipitation. The stability of GPR56-CD9/CD81 complexes in Brij 96, which solubilizes tetraspanin complexes of <4 million daltons (Claas et al., 2001; Stipp et al., 2001b), suggests that a significant fraction of the complexes is stable outside the context of large insoluble raft-like domains. In Triton X-100, a detergent frequently used to prepare classical detergent-insoluble membrane microdomains, the association of GPR56 with CD9 and CD81 was completely disrupted (lane 5).

To test further whether CD9 and CD81 complexes with GPR56 were fully solubilized in Brij 96, biotinylated 293-NFLGPR56 cell lysate was ultracentrifuged at 100,000 × g for 45 min, to pellet detergent-insoluble membrane vesicles before CD81 or GPR56 immunoprecipitation. As shown in Figure 2B, the vast majority of the CD81 complexes in general and the GPR56-CD9/CD81 complexes in particular remained in the supernatant after the 100,000 × g spin (compare lanes 3 and 4 with lanes 1 and 2). Relatively little CD9, CD81, and GPR56 appeared in the 100,000 × g pellet (lanes 5 and 6).
Classical lipid rafts can be disrupted with the cholesterol-depleting reagent, methyl-β-cyclodextrin (MβCD; Yancey et al., 1996). However, CD9 and CD81 association with GPR56 was unaffected by MβCD at 13 mg/ml in cell lysates (Figure 2C). Also, classical lipid rafts float at lower densities in sucrose density gradients. In Brij 96 lysates, these “light membrane fractions” occur at the top of the gradient, in fractions 1–3 (Claas et al., 2001; Stipp et al., 2001b). However, most of the GPR56 and all of the detectable GPR56-CD9/CD81 complexes appeared in higher density fractions (nos. 4–9), as expected for detergent-solubilized “nonraft” proteins (Figure 2D). In sum, the majority of GPR56-CD9/CD81 complexes do not depend upon large, detergent-insoluble membrane microdomains for stability.

**Specific Association of Ga<sub>q/11</sub> and Gβ Subunits with CD9, CD81, and GPR56**

Mass spectroscopic protein sequencing of CD81-associated proteins revealed not only GPR56, but also closely related heterotrimeric G protein subunits, Ga<sub>q</sub> and Ga<sub>o</sub> (from the 40–60-kDa gel region), and Gβ (from the 20–40-kDa gel region; see Table 1). Confirming these results, Ga<sub>q/11</sub> was detected by blotting in CD9 (Figure 3A, lane 2) and CD81 (lane 4) immunoprecipitates, but not in immunoprecipitates of tetraspanins CD63 (lane 3) and CD151 (lane 4), or α2 integrin (lane 6) from a Brij 96 lysate of 293 cells. Unfortunately, the quality of commercially available antisera specific for Ga<sub>o</sub> or Ga<sub>q</sub> did not allow us to assess their individual associations with CD9 and CD81. Using HT1080 cells, which express little or no CD9, we again detected Ga<sub>q/11</sub> specifically associated with CD81 (Figure 3B, lane 3), but not with CD63 or CD151 (lanes 2 and 4). Hence CD9 is unlikely to be required for CD81-Gα<sub>q/11</sub> complex formation. Similarly, we detected Gβ subunit associated with CD81 (Figure 3C, lane 3), but not with CD63 or CD151 (lanes 2 and 4). The Gβ peptide identified by mass spectrometry is a sequence common to β subunits 1–4, and our anti-Gβ antiserum recognizes all four subunits. Thus, the precise identity of the CD81-associated Gβ subunit(s) remains to be determined.

Because CD81 and CD9 associated with both GPR56 and Ga<sub>q/11</sub>, we next tested whether a GPR56-Gα<sub>q/11</sub> complex could be detected. As shown in Figure 3D, we did indeed observe Ga<sub>q/11</sub> associated with both CD81 and GPR56 (lanes 2 and 4), but not with CD151 (lane 3), in a Brij 96 lysate of 293-NFLGPR56 cells. Subsequently, we also confirmed that this specific association of the Gβ subunit with CD9, and GPR56 (see Figure 6, below). Consistent with the fact that no other CD81-associated Ga subunits were identified by mass spectrometry (see Table 1), we did not detect the heterotrimeric G protein subunit, Ga<sub>o</sub>, associated with either CD81 or GPR56 (Figure 3D, lanes 5–8).

**Central Role of CD81 in Promoting/Stabilizing GPR56-Gα<sub>q/11</sub> Association**

The existence of GPR56-CD81, CD81-Gα<sub>q/11</sub>, and GPR56-Gα<sub>q/11</sub> complexes raised the possibility of a GPR56-CD81-Gα<sub>q/11</sub> complex. To test this, biotinylated 293-NFLGPR56 cells were lysed in Brij 96 and immunodepleted with agarose beads alone (mock depletion) or with anti-CD81 or anti-α2 integrin agarose. Depleted pools were then immunoprecipitated with anti-CD81, anti-GPR56 (FLAG), or anti-α2 integrin antibodies. As shown in Figure 4, the CD81 immunodepletion removed all of the detectable CD81, but left behind a substantial pool of GPR56, as expected because of the relatively low fraction of GPR56 associated with CD81. Remarkably, however, this pool of GPR56 contained essentially no associated Gα<sub>q/11</sub>. Thus, all of the Gα<sub>q/11</sub> associated with GPR56 could be removed by CD81 immunodepletion, strongly supporting the existence of GPR56-CD81-Gα<sub>q/11</sub> complexes and suggesting a possible role for CD81 in promoting or stabilizing GPR56-Gα<sub>q/11</sub> association. In the β1 integrin control immunodepletion, neither CD81, nor GPR56, nor the GPR56-associated pool of Gα<sub>q/11</sub> was removed, despite the fact that nearly all of the β1 integrin subunit was depleted. In a converse experiment, depletion of exogenously expressed GPR56 left behind a substantial pool of CD81-associated Gα<sub>q/11</sub> (unpublished data). This was expected because we had previously observed CD81-Gα<sub>q/11</sub> complexes in 293 cells that did not express exogenous GPR56 (Figure 3A).

For a complementary test of CD81 function in supporting GPR56-Gα<sub>q/11</sub> association, we turned to U937 monocytic leukemia cells, selected to lack detectable CD81 expression (Hamaia et al., 2001). We reconstituted these cells with human CD81, NFLGPR56, or both proteins. Flow cytometry...
confirmed the expression of the transduced proteins as well as the relative expression levels of two endogenous proteins, CD97 and CD147 (Table 2). In a separate experiment, we observed no CD9 expression in these cells, using flow cytometry (unpublished data). CD81 and GPR56 (FLAG) immunoblotting also confirmed i) the expression of CD81 specifically in the CD81-transduced cells (Figure 5A, lanes 1 and 3); ii) the expression of GPR56 specifically in the GPR56-transduced cells (Figure 5A, bottom panel, lanes 5 and 6); and iii) the association of CD81 with GPR56 in the dually transduced cells (Figure 5A, top panel, lane 6). In the CD81+ GPR56+ cells, we detected Gαq/11 specifically associated with CD81 (Figure 5B, lane 1) and GPR56 (lane 3), but not with another abundant cell surface protein CD147 (lane 2) or with the LNB-TM7 family member, CD97 (lane 4). The lack of CD97-Gαq/11 association is consistent with our previous observation that CD97 fails to associate with CD81 (Figure 1B).

Next we compared immunoprecipitated GPR56 from lysates of equal numbers of CD81− and CD81+ cells. In the CD81+ cells, dramatically more Gαq/11 coprecipitated with GPR56 (Figure 5C, bottom panel), despite the fact that the mean GPR56 expression level was lower in the CD81+ cells (top panel, and see also Figure 5A, and Table 2). In two separate experiments, the relative amount of Gαq/11 associated with GPR56 was enhanced about fivefold in CD81+ cells, as determined by semiquantitative densitometry (Figure 5D).

### Table 2. Flow cytometry of U937 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GPR56</th>
<th>CD97</th>
<th>CD81</th>
<th>CD147</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937 GPR56</td>
<td>189</td>
<td>102</td>
<td>1</td>
<td>353</td>
</tr>
<tr>
<td>U937 CD81</td>
<td>0</td>
<td>114</td>
<td>502</td>
<td>449</td>
</tr>
<tr>
<td>U937 GPR56/CD81</td>
<td>115</td>
<td>107</td>
<td>285</td>
<td>419</td>
</tr>
</tbody>
</table>

Mean fluorescence intensity units (mFI) after subtraction of background staining from an irrelevant antibody. GPR56 staining was with anti-FLAG antibody.

### Dynamic Regulation of the GPR56-CD81-Gαq/11 Complex: CD81 Antibody Effects

Because the GPR56 ligand has not been identified, it was not feasible to test directly CD81’s role in signaling through GPR56. Therefore, we tested the effects of engaging the GPR56-CD81-Gαq/11 complex with an anti-CD81 antibody, M38. When intact HT1080 cells were treated with the M38 antibody for 15 min before lysis in Brij 96 and CD81 immunoprecipitation (Figure 6A), there was greatly diminished Gαq/11 association with CD81 (compare treated cells in lane 3 to untreated control in lane 1). Pretreating the cells with a different anti-CD81 mAb, JS64, did not reduce the amount of CD81-associated Gαq/11 (lane 4). Furthermore, M38 antibody added after lysis reduced the CD81-Gαq/11 association to a much lesser extent (lane 2). Similar results were obtained in three independent experiments.

We hypothesized that if CD81 is central to GPR56-Gαq/11 association, then perturbing CD81-Gαq/11 interaction should also perturb GPR56-Gαq/11 interaction. Indeed pretreatment of intact 293-NFLGPR56 cells with M38 caused dissociation of Gαq/11 from GPR56 (Figure 6B, lane 3), just as we had observed for CD81. A control anti-CD151 antibody had no effect on GPR56-Gαq/11 association (lane 4). Although the antibody treatment time was brief, it was possible that M38-induced CD81 internalization was the basis for disrupted Gαq/11 interaction. However, using cell membranes (Figure 6B, lanes 5 and 6) or prechilled cells in ice-cold buffer (Figure 6C, lanes 2 and 3), we again found that M38 antibody triggered dissociation of Gαq/11 from GPR56. Thus M38 retained the ability to disrupt the complexes even under conditions where the endocytic machinery had presumably been disabled by subcellular fractionation or was impaired because of cell incubation at 0°C.

M38 treatment of intact 293-NFLGPR56 cells had no effect on the amount of GPR56 subsequently recovered, nor was the amount of CD81 associated with GPR56 reduced (Figure 6D). However, the amount of Gαq/11 associated with the GPR56-CD81 complex was dramatically reduced, even after 5 min of antibody exposure, and continued to decline at the 15-min time point. Thus, unlike the previous immunodepletion experiments, in which the pool of CD81-associated Gαq/11 was removed, leaving behind a pool of GPR56 that did not associate with Gαq/11 (Figure 4), treatment of intact cells with the M38 antibody triggered the loss of Gαq/11 without depleting or disrupting the CD81-GPR56 complex. As shown in Figure 6E, M38 treatment also disrupted association of the Gα subunit with GPR56, CD9, and CD81 (compare lanes 2 and 3, lanes 4 and 5, and lanes 6 and 7 respectively). No Gβ was detected in association with CD151 or α2 integrin, regardless of the presence of M38 (lanes 8–11).

The preservation of the GPR56-CD81 association, even as Gαq/11 dissociated, suggested that Gαq/11 is not required for GPR56-CD81 interaction. To test this further, we immunoprecipitated CD81 complexes from Brij 99 lysates containing increasing amounts of Triton X-100. As shown in Figure 6F, CD81 associations with GPR56 and Gαq/11 decreased in parallel as Triton X-100 concentration increased, until, at 0.5% Triton X-100, the CD81-Gαq/11 association fell off more sharply. This result was again consistent with the GPR56-CD81 association being independent of the presence of Gαq/11.

In control experiments, M38 treatment of intact cells had little or no effect on CD81 association with other cell surface part-
nners, EWI-2, EWI-F, and α3β1 integrin (unpublished data). Thus, M38’s effects on CD81 interactions appear to be relatively specific and provide a rare example of dynamic regulation of a tetraspanin complex. They also provide independent confirmation of the central role of CD81 in supporting GPR56-Gαq/11 association.

Dynamic Regulation of the GPR56-CD81-Gαq/11 Complex: Phorbol Ester Stimulation

Phorbol ester (PMA) treatment triggers internalization and desensitization of several GPCRs (Diviani et al., 1997; Liang et al., 1998; Tang et al., 1998; Orsini et al., 1999; Xiang et al., 2001; Rochdi and Parent, 2003). We therefore tested whether PMA stimulation mobilized cell surface GPR56, and, if so, what effect this had on CD81 and Gαq/11 association. As shown in Figure 7A, PMA triggered a rapid loss of GPR56-FLAG epitope from the surface of U937 cells such that ~30% of the GPR56 was lost by the end of the 20-min PMA stimulation, and ~85% was lost within the following 2 h. In contrast, PMA treatment caused only a modest decrease in cell surface CD81 over a subsequent 2-h interval. The kinetics of the loss of GPR56 were virtually identical in CD81- and CD81+ cells, indicating that CD81 is not required for this type of GPR56 sequestration.

We next examined the effect of PMA-induced GPR56 sequestration on GPR56 association with CD81, Gαq/11, and Gβ. We chose a time point immediately after PMA stimulation, when cell surface GPR56 was still at ~70% of untreated levels. Even at this early time point, a dramatic 78–87% decrease in the amount of CD81 associated with GPR56 had already occurred (Figure 7B, compare lanes 1 and 2). Concurrently, the amount of Gαq/11 and Gβ subunit associated with GPR56 also decreased by 67–70% (compare lanes 7 and 8 and lanes 13 and 14), despite the fact that the amount of GPR56 recovered was not diminished and in fact was elevated (compare lanes 19 and 20). On PMA stimulation, the amount of Gαq/11 (lanes 9, 10) and Gβ (lanes 15, 16) associated with CD81 did not decrease. Small apparent diminutions in Gαq/11 and Gβ levels (lanes 10 and 16) can be almost entirely explained by the reduced recovery of CD81 (compare lanes 3 and 4). Thus, PMA stimulation caused a dramatic loss of cell surface GPR56, likely by GPR56 internalization, with the concomitant dissociation of GPR56 from CD81-Gαq/11-Gβ. As a specificity control, no CD81, Gαq/11, Gβ, or GPR56 coprecipitated with CD147 (lanes 5 and 6, all panels). The lack of GPR56 detected by anti-FLAG blotting of CD81 immunoprecipitates reflects the limited sensitivity of the anti-FLAG antibody in immunoblotting, as discussed in Figure 1D. Altogether, these results reveal a second mechanism whereby the GPR56-CD81-Gαq/11-Gβ complex can be dynamically regulated, and they provide yet more evidence for the central role of CD81 in facilitating the interaction of GPR56 with heterotrimeric G protein subunits.

DISCUSSION

Highly Specific Complexes of GPR56 and Gαq/11 with CD9 and CD81

In the present study, we identified the heptahelical protein GPR56 as well as heterotrimeric G protein subunits Gαq, Gα11, and Gβ as novel CD9 and CD81 partners. These interactions are specific at three levels: i) GPR56 but not CD97, a GPCR from the same subfamily, associated with CD9 and CD81. Hence not all GPCRs will form complexes with CD9 and CD81 to the same extent. ii) CD9 and CD81, but not tetraspanins CD151 or CD63, associated with GPR56 and/or Gαq/11 and Gβ. iii) Heterotrimeric G protein subunit Gαq/11 but not Gαq, associated with CD81 and GPR56. Also, GPR56 associated with CD9 and CD81 to a greater extent than did α6 integrin, even though α6 integrin can associate with CD9 and CD81 in some cell types (Berditchevski et al., 1996). We relied on endogenous expression of CD81 (in most cases) and Gαq/11 (in all cases), and the apparent expression of FLAG-tagged GPR56 was similar to that of the endogenous GPCR, CD97 in U937 cells; thus, the interactions are relevant at physiological protein levels. Although other GPCRs and heterotrimeric G proteins were absent from our initial mass spectrometry screening results, preliminary data from other
Figure 6. An anti-CD81 antibody triggers dissociation of Goq/11, from GPR56-CD81. (A) About 5 × 10^6 HT1080 cells were treated for 20 min at 37°C with 5 μg/ml anti-CD81 mAbs M38 (lane 3) or JS64 (lane 4) or were left untreated (lanes 1 and 2). Cells were rinsed with ice-cold PBS and lysed on ice in 1% Brij 96. After centrifugation, lysates were precleared with mouse IgG directly conjugated to agarose. For the sample in lane 2, 5 μg/ml M38 was added during this preclearing step. After preclearing, JS64 and M38 concentrations in each lysate were adjusted to 5 μg/ml, protein G was added, and CD81 immune complexes were collected. The presence of Goq/11 in each immunoprecipitate was measured by immunoblotting. (B) Top panel: ~7 × 10^6 293-NFLGPR56 cells were treated for 15 min at 37°C with 5 μg/ml anti-CD81 mAb, M38 (lane 3), anti-CD151 mAb, 5C11 (lane 4) or no antibody (lane 2), before rinsing in cold PBS and lysing in 1% Brij 96. GPR56 was immunoprecipitated from the extracts, and Goq/11 was blotted, as in lanes 1–4. (C) 293-NFLGPR56 cells were left untreated (lane 1), treated with M38 at 37°C for 15 min (lane 2), or prechilled on ice and treated with M38 in ice-cold buffer for 15 min (lane 3). Cells were then lysed in Brij 96, and GPR56-Goq/11 association was analyzed as in B. By densitometry, Goq/11 association was reduced by 84% (lane 2) and 63% (lane 3) after correction for small variations in recovered GPR56. (D) About 7 × 10^6 293-NFLGPR56 cells were treated with the M38 antibody for the indicated times (0 min was not treated) before lysis in Brij 96 and GPR56 immunoprecipitation as in B. The levels of GPR56, CD81, and Goq/11 in each immunoprecipitate were assayed by immunoblotting. (E) The indicated proteins were immunoprecipitated from 1% Brij 99 lysates of 293-NFLGPR56 cells that had been treated with M38 anti-CD81 (15 min, at 37°C) or left untreated. Coprecipitating Gβ subunit was detected by immunoblotting. About 2 × 10^6 cell equivalents were analyzed in lanes 2–11, compared with ~2 × 10^7 in lane 1. (F) A clarified Brij 99 extract of ~5.4 × 10^7 biotinylated 293-NFLGPR56 cells was divided into five equal pools, and Triton X-100 was added to the indicated concentrations. After CD81 immunoprecipitation and rinsing with the appropriate detergent mixture, 90% of the bead-bound CD81 immune complexes were resuspended in 1% Triton X-100 (no Brij 99) to elute CD81-associated proteins, and then GPR56 was reimmunoprecipitated and detected by blotting with HRP-ExtrAvidin (top panel). The remaining 10% of the CD81 immune complexes were blotted for Goq/11 (bottom panel).

mass spectrometry experiments suggest that additional tetrarpspin-GPCR complexes exist, with different tetrarpsins perhaps forming specific complexes with different GPCR partners in a cell type–specific manner (T. Kolesnikova, X. Yang, C. Stipp, and M. Flemier, unpublished data). Importantly, GPR56 complexes with CD9 and CD81 appeared in the dense fractions rather than in the light membrane fractions of sucrose gradients, were not disrupted by cholesterol depletion, and did not pellet on centrifugation at 100,000 × g. Hence GPR56-CD9/CD81 complexes are not simply the product of incomplete detergent solubilization, but rather represent discrete, solubilized biochemical entities. It remains to be determined whether CD9 and CD81 complexes with GPR56, Goq/11, and Gβ will overlap with other CD9 and CD81 complexes containing molecules such as TGF-α (Shi et al., 2000), HB-EGF (Iwamoto et al., 1994; Nakamura et al., 1995), and CD19/CD21 (Levy et al., 1998).

A Central Role for CD81 in Facilitating GPR56-Goq/11 Interactions

Several results strongly support the existence of GPR56-CD81-Goq/11 complexes. Immuno depletion of CD81 essentially abolished Goq/11 association with GPR56. Conversely, reexpressing CD81 in CD81– cells dramatically enhanced GPR56-Goq/11 complex formation. Furthermore, anti-CD81 antibody treatment (or harsh detergent) led to separation of GPR56-CD81 from Goq/11, whereas PMA treatment separated GPR56 from CD81-Goq/11. Under no conditions did we observe separation of CD81 from GPR56-Goq/11. Hence CD81 clearly plays a central role in the GPR56-CD81-Goq/11 complex. The physical arrangement of the molecules within the GPR56-CD81-Goq/11 complexes remains to be more precisely defined. Because CD81 may typically exist as a dimer (Kovalenko et al., 2004) and can form an expanded network of interactions with other molecules (Kolesnikova et al., 2004), we suspect that GPR56-CD81-Goq/11 complexes also

Vol. 15, May 2004 2383
exist as part of an expanded network of interactions, rather than as a simple 1:1:1 linear complex. The presence of the G\(\beta\) subunit in GPR56-CD81-G\(\alpha_{q/11}\) complexes is consistent with CD81 promoting GPR56 association with an intact G\(\alpha_{q/11}\) heterotrimeric G protein. Because immunodepletion experiments indicate that the pool of GPR56 associated with G\(\alpha_{q/11}\) and the pool associated with CD81 are one and the same, CD81 may be perfectly positioned to influence the extent of GPR56 coupling to G\(\alpha_{q/11}\) upon activation.

**Dynamic Regulation of the GPR56-CD81-G\(\alpha_{q/11}\) Complex on Intact Cells**

For most tetraspanin complexes, there is little evidence suggesting that they are rapidly altered upon cell stimulation or activation. Our data revealed two different types of dynamic regulation of the GPR56-CD81-G\(\alpha_{q/11}\) complex (Figure 8). First, engaging the complex on intact cells with the anti-GPR56 antibody, M38, triggered the simultaneous loss of G\(\alpha_{q/11}\) and G\(\beta\) from CD81 and GPR56, while maintaining the CD81-GPR56 association. Conceivably, the M38 antibody could be acting as a surrogate ligand, mimicking some aspects of GPCR ligand binding, such as G protein mobilization (Rodbell, 1997) or translocation of GPCR into or out of different cell surface microdomains (Ostrom et al., 2000). Ligand binding frequently triggers GPCR phosphorylation (Pierce et al., 2002); however, we failed to detect any phosphorylation of GPR56 upon M38 treatment of \(^{32}\)PO\(_4\)-labeled cells (unpublished data). Furthermore, M38 did not trigger a redistribution of G\(\alpha_{q/11}\) into or out of detergent insoluble membrane microdomains, and, using cells labeled with \(^{3}H\)inositol, we did not observe an increase in total cellular IP\(_3\) upon M38 treatment (unpublished data). Collectively, these data suggest that the loss of G\(\alpha_{q/11}\) from CD81-GPR56 complexes does not correspond to activation of the G\(\alpha_{q/11}\)-phospholipase C\(\beta\) (PLC\(\beta\)) signaling cascade. However, local activation of G\(\alpha_{q/11}\) or PLC\(\beta\) might be overlooked in whole cell lysates.

The second example of dynamic regulation of the GPR56-CD81-G\(\alpha_{q/11}\) complex resembles the phenomenon of heterologous GPCR desensitization, in which signaling through PKC or PKA pathways can drive desensitization/internalization of non-ligand-bound GPCRs (Ferguson, 2001; Pierce et al., 2002). Indeed we found that PMA stimulation of intact cells resulted in the apparent internalization of GPR56, with concomitant loss of G\(\alpha_{q/11}\) and G\(\beta\) from CD81 and GPR56, while maintaining the CD81-GPR56 association. Conceivably, the M38 antibody could be acting as a surrogate ligand, mimicking some aspects of GPCR ligand binding, such as G protein mobilization (Rodbell, 1997) or translocation of GPCR into or out of different cell surface microdomains (Ostrom et al., 2000). Ligand binding frequently triggers GPCR phosphorylation (Pierce et al., 2002); however, we failed to detect any phosphorylation of GPR56 upon M38 treatment of \(^{32}\)PO\(_4\)-labeled cells (unpublished data). Furthermore, M38 did not trigger a redistribution of G\(\alpha_{q/11}\) into or out of detergent insoluble membrane microdomains, and, using cells labeled with \(^{3}H\)inositol, we did not observe an increase in total cellular IP\(_3\) upon M38 treatment (unpublished data). Collectively, these data suggest that the loss of G\(\alpha_{q/11}\) from CD81-GPR56 complexes does not correspond to activation of the G\(\alpha_{q/11}\)-phospholipase C\(\beta\) (PLC\(\beta\)) signaling cascade. However, local activation of G\(\alpha_{q/11}\) or PLC\(\beta\) might be overlooked in whole cell lysates.

**Are CD9 and CD81 GPCR Scaffolding Proteins?**

A variety of intracellular scaffolding proteins are emerging as potential answers to longstanding questions about how the vast number of GPCRs in the human genome achieve specificity in signaling through a much more limited number of heterotrimeric G proteins (Hall et al., 1999; Pierce et al., 2002). GPCR-binding proteins such as the \(\beta\)-arrestins, PDZ, SH2, and polyproline binding proteins may help create GPCR signaling specificity by engaging additional signaling pathways or localizing GPCR signaling events to specific subcellular sites. The central role of CD81 in promoting GPR56-G\(\alpha_{q/11}\) association argues that CD9/CD81 complexes may have analogous GPCR scaffolding functions. The
apparently low fraction of GPR56 and Goq/11 associated with CD9 and CD81 is not inconsistent with the low fractional association of known scaffolding proteins, such as β-arrestin 1 associating with the ligand-bound GPCR, β2 adrenergic receptor (Luttrell et al., 1999).

Association of GPR56 and Goq/11 with CD81 and CD9 opens up a range of scaffolding possibilities. These tetrarospanins have been linked to several other proteins relevant to G protein signaling, including PKC (Zhang et al., 2001a), the integral membrane metalloproteinase kuzbanian/ADAM 10 (Yan et al., 2002), EGF receptor ligands (Iwamoto et al., 1994; Nakamura et al., 1995; Shi et al., 2000), and phosphatidylinositol 4-kinase (Berditchevski et al., 1997b; Yauch et al., 1998). CD9 and CD81 can functionally link conventional PKC isoforms to α3 or α6 integrins, leading to integrin phosphorylation accompanied by altered cell motility (Zhang et al., 2001a, 2001b). Because these PKC isoforms are major downstream effectors of Goq/11, it is tempting to speculate that CD9/CD81 might likewise promote linkage of GPR56 and Goq/11 to PKC. A further suggestion of a GPCR scaffolding function for CD9/CD81 complexes comes from a recent study of the GPCR-mediated transactivation of the EGF receptor (EGFR; Yan et al., 2002). In this study, the GPCR ligand, bombesin, triggered EGFR phosphorylation and signaling and also promoted CD9 association with kuzbanian/ADAM 10, which can cleave cell surface HB-EGF, itself a CD9-associated protein (Iwamoto et al., 1994; Nakamura et al., 1995). The kuzbanian-mediated shedding of HB-EGF appears to be responsible for EGFR transactivation in this study. Thus, complexes organized by CD9 may be important for promoting a GPCR activation-dependent association between kuzbanian and HB-EGF. Finally, the activation of phosphatidylinositol 4-kinase by GPCR ligands such as mastoparan (Gasman et al., 1998) suggests yet another potential scaffolding function for CD9 and CD81.

Possible Physiological Relevance of GPR56-CD9/81-Gq/11 Complexes

The placement of GPR56 within the LNB-TM7 subfamily of GPCRs provides clues to potential functions in vivo. The 30 or more subfamily members share the unusual property of long extracellular N-termini that contain structural motifs from other protein superfamilies (Stacey et al., 2000; Krasnoperov et al., 2002; Fredriksson et al., 2003). These motifs, which include EGF-like, Ig-like, laminin G domain-like, thrombospondin type I-like, and protocadherin-like domains, suggest that many LNB-TM7 family members may bind to extracellular matrix proteins or cellular counterreceptors, rather than small molecule ligands. By analogy,
GPR56 and its ligand may be involved in cell-cell or cell-substrate interactions. One function of CD9 and CD81 might be to enhance GPR56 ligand binding, in the same way that CD9 association promotes HB-EGF diphtheria toxin binding (Iwamoto et al., 1994) and potentiates juxtaacellular HB-EGF signaling (Higashiyama et al., 1995).

The presence of GPR56 mRNA in hematopoietic and neural precursor cell populations (Terskikh et al., 2001) suggests possible functions in stem cell proliferation or differentiation, and indeed GPR56 expression is selectively high in ventricular zones in the developing CNS (Terskikh et al., 2001), where neural cells are rapidly proliferating. This is potentially interesting in light of dramatic overproduction of glial cells during brain development in CD81-null mice (Geisert et al., 2002). GPR56 expression also persists in several regions of the adult brain (Liu et al., 1999), suggesting possible functions in differentiated neurons or glia as well. GPR56 expression is downregulated on highly metastatic melanoma cells (Zendezman et al., 1999), whereas the loss of CD9 also correlates with increased metastases in many cases (Hemler et al., 1996; Boucheix and Rubinstein, 2001). It remains to be determined whether GPR56 will functionally associate with CD9 or CD81 on metastatic tumor cells or with CD9 during sperm-egg fusion (Le Naour et al., 2000; Miyado et al., 2000) or with CD81 while it is regulating Th2 immune responses (Deng et al., 2002).

In conclusion, we have presented evidence that the LN-B7TM protein, GPR56, and heterotrimeric G protein subunits, Goq, Gαs, Goqα, and Gβγ, associate specifically with CD9 and CD81. The central role of CD81 (and CD9 when present) suggests possible scaffolding functions for CD81 and CD9 in GPR56 signaling. Further, we demonstrated two different ways in which these GPR56-CD81-Goqαγ11 complexes can be dynamically regulated: triggering with an anti-CD81 antibody or stimulating with PMA. Several major questions remain to be answered: can functional coupling of GPR56 to Goqαγ11 be established? What role might CD9 and CD81 play in GPR56-mediated signaling events? Are other CD9/CD81 partners, such as α3 integrin or EWI proteins, affected by GPR56 activation? The answers to these questions await the identification either of the GPR56 ligand or of other CD9/CD81-associated GPCRs whose ligands are already known.

ACKNOWLEDGMENTS

The authors are grateful to S. Levy, Stanford University, for providing CD81-negative U937 cells and to W.S. Lane, R. Robinson, and K. Pierce (Harvard Microchemistry Facility) for HPLC, mass spectrometry, and peptide sequencing. We also thank Drs. Fabio Re for a critical reading of the manuscript. This work was supported by grants from the Claudia Adams Barr Foundation and the Medical Foundation (Charles A. King Trust Fellowship; to C.S.S.), and work was supported by grants from the Claudia Adams Barr Foundation and the Medical Foundation (Charles A. King Trust Fellowship; to C.S.S.), and National Institutes of Health Grant CA86712 (to M.E.H.).

REFERENCES


