Signal transfer between the protease-activated PAR1 thrombin receptor and membrane-associated heterotrimeric G proteins is mediated by protein-protein interactions. We constructed a yeast signaling system that resolves domain-specific functions of binding from coupling in the Ga subunit. The endogenous yeast Ga subunit, Gpa1, does not bind to PAR1 and served as a null structural template. N- and C-terminal portions of mammalian Gi2 and G16 were substituted back into the Gpa1 template and gain-of-function assessed. The C-terminal third of Gia6 but not of Gia2 provides sufficient interactions for coupling to occur with PAR1. The N-terminal two-thirds of Gia2 also contains sufficient determinants to bind and couple to PAR1 and overcome the otherwise negative or missing interactions supplied by the C-terminal third of Gpa1. Replacement of the N-terminal α-helix of Gia2, residues 1–34, with those of Gpa1 abolishes coupling but not binding to PAR1 or to βγ subunits. These data support a model that the N-terminal αN helix of the Gia subunit is physically interposed between PAR1 and the Gβ subunit and directly assists in transferring the signal between agonist-activated receptor and G protein.

Thrombin plays a central role in blood coagulation and in the cellular response to injury, inflammation, and wound repair. Many of these complex cellular events are initiated by thrombin cleavage of the protease-activated receptor-1 (PAR1) at residues Arg41-Ser42 located within the N-terminal extracellular domain. The new N terminus, Ser42-Phe-Leu-Leu-Arg-Asn47, serves as an intramolecular ligand that binds and activates the body of the PAR1 thrombin receptor (1–3). Peptides corresponding to the freshly cleaved N terminus can also activate PAR1 by an intermolecular mechanism (1). A number of different guanine nucleotide-binding (G) protein α-subunits have been shown to couple to PAR1, including members of the Ga, Gia2/6 and Gia2/13 families, but the relative importance of these interactions is not well understood (4–8). Recent PAR gene knockout experiments in mice and cloning of two additional thrombin receptors (PAR3 and PAR4) and trypsin/tryptase receptor (PAR2) have shown that different tissues possess a distinct complement of protease receptors that together determine their respective responses to a variety of proteolytic inputs (9–13).

A divergence point in protease receptor signaling is that activation of any particular Gelβ complex will produce two effectors, namely activated Ga and free βγ (14). For instance, since phospholipase C-β can be potentially activated by either Ga or βγ, it is conceivable that non-Ga6 subunits may serve the negative function of sequestering βγ to prevent constitutive activation of the phospholipase C-β pathway (15). In platelets, it now seems likely that Ga3 is the primary effector for phospholipase C-β rather than free βγ. This was demonstrated in Ga3 gene knockout mice (7), whose platelets lack thrombin-dependent phosphoinositide responses or the ability to aggregate. The Ga3 (-/-) platelets retain partial function since they can undergo thrombin-dependent shape change. Therefore, other Ga subunits such as Ga2 and Ga12/13, must necessarily serve as primary effectors for the dramatic cytoskeletal rearrangements that are a prerequisite for platelet aggregation (16).

Other functions of platelet thrombin receptors are influenced by Ga-dependent depression of cAMP levels. The cAMP levels in platelets modulate the activity of platelets; high cAMP levels dampen the response of platelets to agonists and cause attenuation of intracellular Ca2+ levels (17) in part by stimulating Ca2+ efflux by activation of the plasma membrane Ca2+-ATPase (18). The role of Ga in PAR1 responses has been shown by specific pertussis toxin blockade of Ga which significantly reduces SFLLRN-stimulated GTPase activity (19). It is not known how the Ga family members individually regulate adenyl cyclase, however, the relative abundance is Ga2 >> Ga3 >> Ga1 in platelets (20).

One of the major unanswered questions of thrombin receptor signaling is why does PAR1 couple to only a subset of the available G protein partners in a given cell? Presently, there is limited information regarding the molecular determinants of PAR1-G protein interactions. A single study by Coughlin and colleagues (21) involved replacing internal cytoplasmic loops of the Gα2-coupled β2-adrenergic receptor and the Gα12-coupled dopamine D2 receptor with the analogous loops from PAR1. They found that chimeric β2-adrenergic and dopamine D2 receptors bearing the second intracellular loop (22) from the PAR1 acquire Gα12-coupling capability. This led them to conclude that the PAR1 i2 loop may provide “coupling instructions” for Gαi specificity in different receptor contexts but specific PAR1-Gi subunit interactions have yet to be elucidated. With limited high resolution structural data available for receptor-G protein interfaces (22), most of the focus on the general molecular determinants of coupling preferences centers on mutagenesis of receptor i3 loops and the extreme C-terminal residues of Ga subunits (23). For instance, point mutations in the i3 loop of PAR1i2 may provide “coupling instructions” for Gai specificity in different receptor contexts but specific PAR1-Gi subunit interactions have yet to be elucidated. With limited high resolution structural data available for receptor-G protein interfaces (22), most of the focus on the general molecular determinants of coupling preferences centers on mutagenesis of receptor i3 loops and the extreme C-terminal residues of Ga subunits (23). For instance, point mutations in the i3 loop of
the human muscarinic m2 receptor can compensate for point mutations at C-terminal residues of Go (24) consistent with direct intermolecular contact occurring between these regions. Because there are now high resolution x-ray structures of Gafy heterotrimer (25, 26), it is possible to map the solvent-accessible surface of the G protein for the complicity of specific residues in receptor binding and activation. The first such study conducted on a large scale consisted of 100 alanine-substituted solvent-exposed residues of G that were tested biochemically for impaired interactions with rhodopsin (27). Only 9 of the 100 substituted residues were believed to affect solely receptor binding. These sites mapped to the C-terminal 41 amino acids of G, which contains the β7/α7 motif and crucial 11-residue C-tail which are known to mediate receptor-Ga interactions (28). A genetic study of the C-terminal 50 residues of the yeast Go subunit, Gpa1, correlated well with the mammalian data (29). The potential yeast receptor-Gpa1 interface mapped to 4 of the same 9 residues that were identified from the Gpa1 and Gi2 vectors, and subcloning them into pPGK. p195-Gpa1-16 expresses a chimera comprising residues 1–212 of GpA joined to residues 330–472 of Gpa1 (34). The pPGK-Gα-Gpa1 vector expresses a chimera comprising residues 1–50 of the yeast Gα contact surface shares similar architectural features between yeast and mammals.

Given the inherent difficulty in studying structure-function relationships of specific PAR1-G protein complexes in mammalian cells, we developed a more simplified receptor-G protein yeast model system (30–32) that could be used to elucidate mechanistic details of PAR1-G protein coupling. In the studies presented here, we have replaced the endogenous yeast receptor with the human PAR1 thrombin receptor and obtained high affinity agonist binding and functional coupling to co-expressed G proteins. Determinants of PAR1-G protein coupling are separable from binding activity and are found throughout the large interface that encompasses both N- and C-terminal regions of the Go subunit. Our data are also consistent with a model that the N-terminal αN helix of the Ga subunit inserts between PAR1 and the Gaβ subunit and mediates binding and coupling between agonist-activated receptor and G protein. These genetic and biochemical studies further underscore the advantage of using yeast where one can reconstitute a simple signaling unit consisting of ligand-receptor-G-protein that can be tested for functional properties in isolation from all other mammalian proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin (3432 NIH units/mg) was from Haematologic Technologies (Essex Junction, VT). Restriction enzymes and Vent DNA polymerase were from New England Biolabs (Beverly, MA).

**Plasmid Construction**—The stε2::URA3-his3G disruptor construct, pSte2KO, was assembled from 5’ (1480 bp) and 3’ (700 bp) flanking sequences of STE2 with the 5000 bp his3-Gpa1-Kan-his3G fragment from pSE1076 (gift of E. Elion) inserted between them in pUC19. The STE2 expression vector, p112-H6T7Ste2a2300, is derived from the 2 μm TRP1 plasmid, YEp112 (33). The 5’-untranslated region of STE2 was amplified by PCR to create a 544-bp HpaI-XbaI fragment that was placed into corresponding sites of pUC19 and designated p19-5’UTRH6. The fragment of the STE2 gene encoding amino acid residues Ala1 to Thr187 was amplified by PCR and placed into the XbaI-KpnI sites of p185-3’UTR6 (32) to create p19-H6T7Ste2a2300. pUC19 was then used to amplify 200 bp of the 3’-untranslated region of STE2. This 200-bp fragment was ligated into Kpn1-EcoRI sites of p19-H6T7Ste2 to create p19-H6T7Ste2a2300. The 1680-bp HpaI-EcoRI fragment was then shuttled into the corresponding sites of YEp112 and YEp195 (2 μm URA3 (33)) to create p112-H6T7Ste2a2300 and p195-H6T7Ste2a2300. The p112-STE2 vector contains the 2010-bp HpaI-EcoRI wild-type STE2 fragment that was amplified by PCR.

The thrombin receptor expression vector, p112-H6PAR1Δ379 was created by inserting the region of hPAR1 encoding residues Leu377-Glu379 into the BamHI and KpnI sites of p112-H6T7Ste2a2300. PCR was used to amplify the cDNA of hPAR1 subcloned into the SallI sites of Bluescript (gift of S. Coughlin). This BamHI-KpnI fragment was subcloned into the analogous sites of p12-H6T7Ste2a2300 to make p112-H6PAR1Δ379. An extra two codons (Met377–His379) were added to the C terminus to accommodate a Kpn1 restriction site.

The hPAR1 gene comprising residues Leu374-Tyr379 was amplified with PCR primers (+) 5’T-CTCTTGGGATCCAGCCAGGCGAGCGTTGACGC-3’ and (−) 5’GAACTTGGGGATCCAGCCAGGCGAGCGTTGACG-3’, respectively. The 5’-untranslated region of PAR1 was amplified from the Hn11i-PsiI sites of p19H9T7Ste2a2300 (1680-bp H6T7Ste2a2300 fragment subcloned into the Sphi-EcoRI sites of pUC19). The 949-bp Pest-KpnI fragment was then subcloned into the corresponding sites of p112-H6PAR1Δ379 to create p112-H6PAR1. This PAR1 construct has the 5’-terminal amino acids Lys371–Lys379 replaced with Gln371–Lys379.

The high copy number yeast expression vector pRS426-GPA1 (URA3 2 μm) is the 1900-bp EcoRI fragment of GPA1 with flanking 5’ and 3’ GPA1 regulatory elements subcloned into pRS426 (gift of S. DeSimone and J. Kurjan). The pPGK-Gα-Gpa1 vector expresses full-length rat Gα and the pPGK-Gα-Gpa1 vector expresses a chimera comprising residues 1–212 of GpA joined to residues 330–472 of Gpa1 (34). pPGK-aN-12 expresses a chimera that fuses residues 1–42 of residues 35–355 of Gα. This was made by PCR amplification of the respective regions from the Gpa1 and Gα vectors, and subcloning them into pPGK. p195-Gpa1-16 expresses a chimera encoding residues 1–329 of GpA joined to residues 329–374 of Gα under the control of the Gpa1 promoter in the 2 μm URA3 plasmid, YEp115 (33). This was made by PCR amplification of the corresponding Gpa1 coding sequence plus 200 bp of the 5’-untranslated region to the corresponding Gα sequence, and subcloning into YEp115. p195-Gpa1-16 expresses a chimera encoding residues 1–329 of GpA joined to residues 220–374 of Gα, under the control of the Gpa1 promoter. p195-Gpa1–16 was made by replacement of the Gα sequence in p195-Gpa1–12 with the sequence encoding residues 220–374 of Gα. Gα was amplified from the pCIGS16 template by PCR (gift of M. Simon).

**Construction of Yeast Strains**—The yeast strains (Table I) were derived from either W303a or a protease-deficient strain, BJ2168. The chromosomal STE2 allele was deleted by homologous recombination (35) at 5’- and 3’-flanking regions with pStε2KO. The BamHI-PvuII fragment of pStε2KO containing stε2his3G-URA3-his3G was used to transform YK957 and BJ2168, and ura3 derivatives were recovered by counterselection on 5-fluoroorotic acid media (36) to yield KY11 and KY15, respectively. The chromosomal Go gene, Gpa1, was deleted in strain EY1282 (gift of E. Elion) using the knockout construct pSK-IPCR-URA3 (gift of S. DeSimone) digested with EcoRI. A ura3 derivative was made by counterselection on 5-fluoroorotic acid media by previously published methods (37) to make KY187 in far1-deficient genetic backgrounds.

**by Sequestration Determinations in Yeast—**Yeast strain KY187 (gpa1 ura3 his3 FUS1-HIS3) carrying a 2-μm URA3 vector with a Gα subunit gene was grown in synthetic dextrose media deficient for uracil at 250 rpm and 30 °C to ~1 × 10⁶ cells/ml at A600 = 0.4. Cells were pelleted, washed in sterile distilled water, and resuspended to a concentration ~2 × 10⁶ cells/ml in synthetic dextrose media deficient for uracil and histidine. The cells were grown as above for absorbance at 600 nm for 2–3 days. Slow growing cultures were split 2-fold, and histidine was added to a final concentration of 20 mg liter⁻¹ to one set of cultures. Growth and absorbance assays were continued to stationary phase for the cultures with histidine.

**Expression of PAR1 and G Proteins in Yeast—** Cultures (5 ml) of yeast strains harboring Hs7T7-tagged PAR1 expressed from high copy number (2 μm TRP1) vectors were grown to saturation in CAA-TRP media (1 liter contains 6 g of casamino acids, 2% glucose, 1.7 g of yeast nitrogen base, 5 g of ammonium sulfate, 40 mg of adenine, and 20 mg of uracil) at 30 °C. If coexpression of PAR1 and G protein was required, the G-protein was expressed from a second vector (2 μm URA3) using media lacking uracil and tryptophan. A dilution of 1:1000 of the overnight culture was made into fresh CAA-TRP (or CAA-TRP-URA) media and grown at 30 °C to a density of 1–1.5 A600. All further steps were carried out at 4 °C. Cells from 2-liter cultures were harvested by centrifugation for 30 min at 3,300 × g, and resuspended in 200 ml of lysing buffer (20 mM KPO4, pH 7.0, 1 mM MnCl2, 10 mM EDTA, 1% protease inhibitor mixture (38)). Glass beads (0.5 mm) were then added to the cell suspension (50% v/v) and cells were disrupted in a Branson sonicator with an ice-water jacket. The cell lysate was centrifuged for 30 min at 8,000 × g and the supernatant (180 ml) subjected to ultracentrifugation at 150,000 × g for 1 h. The crude membrane pellets were resuspended in 10 ml of storage buffer (20 mM KPO4, pH 7.5, 100 mM NaCl, 100 mM glycerol) by sonication for 1 min at a setting of 4–7 on a Branson sonicator and stored as 1-ml aliquots at −80 °C. Yeast membrane
proteins were maintained at 10–15 mg/ml for stability.

**Ni-chelation Chromatography of His<sub>6</sub>-tagged PAR1-G Protein Complexes**—A 5-ml iminodiacetic acid (IDA)-Sepharose column (Novagen) was charged with 10 ml of 100 mM NiSO<sub>4</sub> and equilibrated with 50 ml of 50 mM Bicine (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9). Typically, 12 mg of crude yeast membrane protein containing His<sub>6</sub>-PAR1-G-protein was applied to the IDA affinity column and recycled for 2 h at 30 °C. These dried fractions were reconstituted in Laemmli sample buffer and resolved by 10% SDS-PAGE and Western analysis.

**Radioligand Binding Assays**—The high affinity peptide agonist, SFLLRN-NH<sub>2</sub>, was a gift from J. Swank (30). Compound 56 was iodinated on tyrosine and purified to radioactivity purity by high performance liquid chromatography by previously described methods (40). The 125<sup>i</sup>-labeled peptide was dissolved in 10% dimethyl sulfoxide, 90% 50T/0.1C (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.1% CHAPS, w/v) as a 10 μM stock solution just prior to use. TRAP peptide (SFLLRN-NH<sub>2</sub>), and NAc-Ala-(pF-Phe)-Arg-cyclohexylalanine-homoarginine-Tyr-NH<sub>2</sub>) was a terminal peptide, KENLKDCGLF, which reacts with G<sub>i</sub> (43). Immunoblotting was done as described previously (44). The Gpa1-Ab is a rabbit polyclonal antibody made against full-length Gpa1 (45). In flow cytometry experiments, yeast cells were fixed in 5% formaldehyde for 2 h at 25 °C prior to fluorescent staining. After 2 successive washes with WB2 (100 mM K<sub>2</sub>HPO<sub>4</sub>, 100 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 M sorbitol), 10<sup>7</sup> cells were resuspended in 1 ml of WB containing 88 units of Lyticease (Roche Molecular Biochemicals) and incubated for 30 min at 30 °C in a roddom to remove the yeast cell wall and form spheroplasts. Intact spheroplasted cells were stained with either the T7-Ab (final concentration 2 μg/ml; Zymed Laboratories Inc.) or goat anti-mouse IgG-fluorescein isothiocyanate (10 μg/ml; DAKO) or goat anti-rabbit IgG-fluorescein isothiocyanate (6 μg/ml; Zymed Laboratories Inc.). Cells were analyzed for fluorescence with a FACScan flow cytometer (Beckton Dickinson).

**RESULTS AND DISCUSSION**

**Expression and Thrombin Cleavage of PAR1 in Yeast Membranes**—PAR1 is a type I integral membrane protein which has a cleavable N-terminal hydrophobic signal sequence that properly orients the receptor in the lipid bilayer (1). The type III endogenous Ste2 α-mating factor receptor uses the first transmembrane α-helix as a signal peptide which is not cleaved by a

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**Table I**

**Yeast strains**

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<td>MATα STE2 GPA1 ura3–1 leu2–3,112 trp1–1 his3–11,15 ade2–1 Gal&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>BJ2168</td>
<td>MATα STE5 GPA1 leu2 trp1 ura3–52 prb1–1122 pep4–3 prr1–407 gal2</td>
<td>R. Rothstein</td>
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**Isogenic derivatives of W303a**

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<td>KY33</td>
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**Isogenic derivatives of BJ2168**

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</tr>
<tr>
<td>KY34</td>
<td>steΔ2 + p112-H6T7Ste23300</td>
<td>This study</td>
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signal peptidase (46). To assure correct topology and stable expression, we converted PAR1 from a type I to a type III membrane protein. The hydrophobic signal sequence was replaced with a hydrophilic His6-T7 tag at the N terminus of PAR1 (H6PAR1). PAR1 was expressed in a high copy number vector under the transcriptional control of the PAR1 (H6PAR1). PAR1 was expressed in a high copy number vector (260–425). The amino acid residue numbers starting with Leu38 are those of the human PAR1. The bottom is anti-SFLLR and anti-T7 Westemns of yeast extracts separated by 10% SDS-PAGE. Lanes 1, 2, and 9 represent yeast extracts from KY51 (Ste2). Lanes 3–8 represent yeast extracts from KY69 (H6PAR1). Lanes 10–13 represent yeast extracts from KY70 (H6PAR1Δ379). The thrombin receptors and yeast mating factor receptor (Ste2) were expressed on a high copy number vector (2 μm KY69 (H6PAR1)). Yeast protein extracts were prepared and probed with a monoclonal antibody (SFLLR-Ab) directed against PAR1 residues Ser42–Phe55. As shown in Fig. 1, lane 5, H6PAR1 localizes to the membrane fraction (UP) and has a relative molecular mass of 34 kDa. The SFLLR-Ab also stains three endogenous yeast proteins migrating at 66, 49, and 46 kDa. Noncovalent higher order H6PAR1 species such as dimer (70 kDa) and tetramer (150 kDa) resist dissociation by 1% SDS as has been observed with other receptor species (47) but can be minimized by the inclusion of 4 M urea in the sample loading buffer. The arrows indicate monomeric (m) and dimeric (d) receptor species.

One test of PAR functionality is the ability of the cognate protease to recognize and cleave the receptor at the correct cleavage site. As shown in Fig. 1, we demonstrated that yeast-expressed PAR1 can be cleaved at the Arg41–Ser42 peptide bond by nanomolar thrombin. Treatment of H6PAR1 with 5 nM thrombin results in essentially complete loss of the T7-epitope (Fig. 1, lane 8) and the expected slight downward shift in mass is detected by the SFLLR-Ab (Fig. 1, lane 6). Likewise, cleavage of H6PAR1Δ379 at the Arg63 site with 1 nM thrombin causes >97% loss of the T7-epitope (Fig. 1, lane 13). Further characterization of plasma protease cleavage sites and cleavage kinetics of the yeast-produced receptor are presented in a separate report (42).

**Cellular Localization of PAR1 in Yeast**—Flow cytometry was used to determine whether PAR1 is targeted to the yeast plasma membrane and in the proper orientation. As positive and negative controls, we first examined the localization of H6T7Ste2 (KY34) and H6Ste2 (KY27) which lacks the T7 epitope. As shown in Fig. 2C, the median fluorescence intensity of KY34 increased 1.5-fold relative to KY27 in the absence of Tween 20. When these cells were permeabilized with 0.1% Tween 20, the median fluorescence intensity increased to 2.6-fold relative to KY27. Therefore, the further increase in fluorescence of the Tween 20 permeabilized KY34 cells relative to the intact KY34 cells indicates that the highly expressed H6T7Ste2 is distributed both on plasma membrane and within membrane compartments located in the cytoplasm. Examination of T7-Ab-stained KY34 by in situ immunofluorescence showed that H6T7Ste2 stains as a uniform coating on the plasma membrane and as bright-stained objects within the cytoplasm (data not shown). The cellular distribution of
PAR1 Thrombin Receptor-G Protein Interactions

In order to determine whether the yeast Gpa1 subunit does not copurify with H6PAR1. Since the genomic GPA1 allele gives low levels of Gpa1 protein expression, we overexpressed Gpa1 5–10-fold over background levels using a high copy number plasmid (Fig. 3, lane 14). As shown in Fig. 3, lane 15, even these high levels of Gpa1 do not bind to H6PAR1. Since the full-length Gpa1 does not bind to H6PAR1 it was used as a null structural template in the subsequent chimera studies. We could then add back portions from authentic couplers such as G_12 or G_16 and test for gain of binding in order to delineate domain-specific functions within the Gα subunits. The chimeric Gα subunits were constructed using C- and N-terminal portions of mammalian G_12, G_16, and the endogenous yeast Gα: Gpa1-12, Gpa1-16, and G_16-gpa1 (Fig. 6). The Gpa1-i2 chimera comprises residues 1–329 of the yeast Gpa1 and residues 213–355 of G_12 (34). The Gpa1–16 chimera comprises residues 1–329 of Gpa1 joined to residues 220–374 of G_16. The reverse G_12-gpa1 chimera comprises residues 1–212 of the G_12 joined to residues 330–472 of Gpa1. The chimeric junction (Fig. 6) is located at the highly conserved switch II region (25). To simplify interpretation of the Western data, we deleted the genomic GPA1 allele (Fig. 3, lane 16) and expressed the Gpa1 chimeras in the gpa1 null background. The chimeric Gα proteins, Gpa1-12 and Gpa1-16, were expressed in yeast, and both co-purify with H6PAR1 (Fig. 3, lanes 10, 11, and 13, respectively). Therefore, the C-terminal third of G_12 or G_16 (48) is sufficient to confer binding to PAR1 although the majority of the chimera is Gpa1. The reverse chimera, G_12-gpa1, which had been reported in earlier studies to be expressed in yeast (34) is not detected by the AS-Ab (Fig. 3, lane 13) but was observed by the Trp-Ab (Fig. 3, lane 14). Membrane fractions containing H6PAR1-G protein complexes were bound to Ni-IDA null background. The chimeric Gα subunits were constructed using C- and N-terminal portions of mammalian G_12, G_16, and the endogenous yeast Gα: Gpa1-12, Gpa1-16, and G_16-gpa1 (Fig. 6). The Gpa1-i2 chimera comprises residues 1–329 of the yeast Gpa1 and residues 213–355 of G_12 (34). The Gpa1–16 chimera comprises residues 1–329 of Gpa1 joined to residues 220–374 of G_16. The reverse G_12-gpa1 chimera comprises residues 1–212 of the G_12 joined to residues 330–472 of Gpa1. The chimeric junction (Fig. 6) is located at the highly conserved switch II region (25). To simplify interpretation of the Western data, we deleted the genomic GPA1 allele (Fig. 3, lane 16) and expressed the Gpa1 chimeras in the gpa1 null background. The chimeric Gα proteins, Gpa1-12 and Gpa1-16, were expressed in yeast, and both co-purify with H6PAR1 (Fig. 3, lanes 10, 11, and 13, respectively). Therefore, the C-terminal third of G_12 or G_16 (48) is sufficient to confer binding to PAR1 although the majority of the chimera is Gpa1. The reverse chimera, G_12-gpa1, which had been reported in earlier studies to be expressed in yeast (34) is not detected by the AS-Ab or Gpa1-Ab. However, the G_12-gpa1 reverse chimera was found to couple efficiently to PAR1 by functional assays (see below).

Thrombin Receptor Agonist Peptide (TRAP) Binding to PAR1-G Protein Complexes—Next, we ascertained whether the heterologously produced PAR1-G protein complexes could bind SFLLRN peptide ligand. We prepared a high affinity radioligand, 125I-SFLLRN, that has an EC₅₀ of 30 nM for the human platelet PAR1 (39). Membrane fractions containing H6PAR1-G protein complexes were bound to Ni-IDA beads and washed extensively to reduce nonspecific binding of the hydrophobic 125I-SFLLRN peptide ligand. As shown in Fig. 4, SFLLRN peptide displaces 125I-SFLLRN from H6PAR1/G_12 and H6PAR1/G_16-Gpa1 with IC₅₀ values of 2–3 μM (Table II). The maximal amount of bound 125I-SFLLRN is in the range of 10–13 pm (Table II). Cells expressing Gpa1 along with H6PAR1 gave an IC₅₀ value of 54 μM for the SFLLRN ligand. Since the endogenous Gpa1 does not bind H6PAR1 under these conditions, this 22-fold poorer IC₅₀ value probably reflects the low affinity state of PAR1 in the absence of bound G protein. For comparison, 10-fold shifts in Kᵩ were seen with α-factor peptide binding to Ste2 when GTPγS was added to dissociate bound Gpa1/βγ (49). In a negative control experiment, NαC-TRAP peptide, which does not bind the receptor, could not displace 125I-SFLLRN from H6PAR1/G_12 at concentrations as high as 250 μM. In a second negative control experiment using yeast extracts that lacked the thrombin receptor, H6T7Ste2 was overexpressed and membrane extracts bound to Ni-IDA beads as before. In this case, very low levels (0.2 pm) of
of protein.

Now that we had detected binding between PAR1 and Gi2, we pressed in Yeast—

SFLLRN peptide (Fig. 4).

Boundmin, and Hill coefficient, as described under “Experimental Procedures.” Loaded material is designated Ni+ and material eluted from the Ni-IDA column with 100 mM imidazole is designated Ni.

nonspecific background binding of 125I-56 bind to Ni-IDA-H6T7Ste2 and this level is unaffected by addition of GDP displaces GTP-γ-35S with EC50 values ranging from 1.8 to 4.4 μM (Table II). Addition of GDP displaces ≈96% of bound GTP-γ-35S from the SFLLRN-activated H6PAR1-Gi2 complex (Fig. 5C).

Similar reductions are seen for the H6PAR1-Gi2-Gpa1 and H6PAR1-Gpa1–16 complexes (data not shown). There is no SFLLRN-dependent binding of GTP-γ-35S to Gpa1 coexpressed with H6PAR1 (Fig. 5C), as expected since Gpa1 does not bind PAR1. Coupling by Gt-gpa1 shows that the N-terminal two-thirds of Gi2 provide sufficient functional interactions with PAR1, and that the C terminus of Gpa1 can substitute for Gi2 to provide the essential C-terminal coupling determinants. Conversely, the Gpa1–16 chimera couples to PAR1 thus demonstrating that the C terminus of G16 can also provide sufficient positive interactions to dominate the otherwise deficient interactions occurring at the N terminus of Gpa1 (48). Most surprisingly, PAR1 does not couple to the bound Gpa1-i2 chimera despite having the appropriate C-terminal β6/α6 motif (Fig. 6). This is also a first indication that N-terminal regions of the Gα subunit can supersede C-terminal regions in the control of G protein-PAR1 coupling.

The αN helix is the prime candidate within the N-terminal two-thirds of the Gα subunit for mediating coupling between the receptor and G protein. This αN helix is the site of N-terminal lipid modification of the Gα subunit and is expected to orient the G protein heterotrimer in juxtaposition to the plasma membrane and the intracellular loops of the G protein-coupled receptors (25). Early support for this model was provided by Hamm et al. (50) who demonstrated that an 8–23 Gt peptide blocked full-length Gt from binding rhodopsin. The first direct evidence for this topological model was provided by Neubig and colleagues (51) who detected cross-linking between intracellular loops from the α2-adrenergic receptor and the G protein. This shows that the receptor and G protein. This assay is analogous to other analogous parameters derived from mammalian systems. The SFLLRN-dependent nucleotide exchange EC50 values which

125I-56 bind to Ni-IDA-H6T7Ste2 and this level is unaffected by SFLLRN peptide (Fig. 4).

Functional Coupling of PAR1-G Protein Complexes Expressed in Yeast—Now that we had detected binding between PAR1 and Gα subunits, we tested whether the complexes would undergo nucleotide exchange (19, 41) in response to the SFLLRN agonist. We devised a GTP-γ-35S nucleotide exchange assay that was optimized for our His-tagged PAR1. As shown in Fig. 5, addition of SFLLRN to the H6PAR1-Gi2–16, H6PAR1-Gi2-Gpa1, or H6PAR1-Gi2 complexes stimulate binding of GTP-γ-35S with EC50 values ranging from 1.8 to 4.4 μM (Table II). Addition of GDP displaces ≈96% of bound GTP-γ-35S from the SFLLRN-activated H6PAR1-Gi2 complex (Fig. 5C).

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![Fig. 3. Purification of His6-tagged PAR1-G protein complexes by Ni-chelate chromatography. Top, Western blots (10% SDS-PAGE) of protein chromatographed on a 5-ml Ni-IDA column using antibodies directed against the C-tail of Gi2 (AS-Ab) or against whole Gpa1 (Gpa1-Ab) as described under “Experimental Procedures.” Loaded material is designated Ni+ and material eluted from the Ni-IDA column with 100 mM imidazole is designated Ni.](image)

![Fig. 4. Displacement of high-affinity radioligand 125I-56, by PAR1 peptide ligands. Yeast membrane proteins were bound to Ni-IDA beads and washed with 0.1% CHAPS-containing buffer. Protein-Ni-IDA beads (25 μl/assay) were incubated with 0.7–0.9 nM 125I-56, for 30 min, 4 °C, in the presence of varying concentrations of TRAP peptide as described under “Experimental Procedures.” Membrane proteins were from strains: , KY91 (H6PAR1/Gi2-Gpa1); , KY92 (H6PAR1/Ga2); , KY64 (H6PAR1/Gpa1); , KY33 (H6T7Ste2Δ300/Gpa1); , KY92 (H6PAR1/Ga2) titrated with NAc-TRAP instead of TRAP. The non-specific background binding of 125I-56 to Ni-IDA beads alone was subtracted from each data point. The binding curves were fit by non-linear regression using KaleidaGraph and allowing IC50, Boundmax, and Hill coefficient, n, to float. The best values and standard errors for IC50, and Boundmax (pm) are listed in Table II. Each binding curve is the average of triplicate samples and is representative of three independent experiments.](image)
Individual $^{125}$I-56 ligand binding experiments are shown in Fig. 4. GTP-$\gamma$-35S binding experiments were conducted as described in the legend to Fig. 5.

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Receptor</th>
<th>G protein</th>
<th>SFLLRN IC$<em>{50}$ or EC$</em>{50}$</th>
<th>Maximal bound$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-56</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$gpa1</td>
<td>3 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>$^{125}$I-56</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$</td>
<td>2 ± 1</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S + NAc-TRAP$^b$</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$gpa1</td>
<td>&gt;1 mM</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$</td>
<td>64 ± 50</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S</td>
<td>Ste2</td>
<td>Gpa11-16</td>
<td>1.8 ± 0.9</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$</td>
<td>4.4 ± 0.9</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S + GDP</td>
<td>PAR1</td>
<td>G$_{\alpha_2}$</td>
<td>&lt;500</td>
<td>0.07</td>
</tr>
<tr>
<td>GTP-$\gamma$-35S</td>
<td>PAR1</td>
<td>Gpa1-12</td>
<td>&gt;1000</td>
<td>0.3</td>
</tr>
<tr>
<td>GTP-$\gamma$S</td>
<td>PAR1</td>
<td>Gpa1</td>
<td>&gt;1000</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$^a$ Referred to as Bound$_{\text{max}}$ in Figs. 4 and 5.

$^b$ Titration was performed with NAc-TRAP instead of SFLLRN and did not displace bound $^{125}$I-56.

$^c$ 10 $\mu$M GDP was included in reaction mixture.

we obtained for individual G$_{\alpha_2}$, Gpa1-G$_{16}$, and G$_{\alpha_2}$-Gpa1 subunits coupled to PAR1 agree nicely with those values derived from platelets. Seiler et al. (19) obtained EC$_{50}$ values of 1–3 $\mu$M for GTPase activation with platelet membranes. Since platelet extracts contain at least 9 $\alpha_2$ subtypes (52) these EC$_{50}$ values must be considered to be an aggregate measurement although inhibition with pertussis toxin indicated that the majority of the GTPase activity derives from G$_{\alpha_2}$ subunits. In the studies presented here, we have made the first quantitative measurements of coupling between isolated PAR1-G protein pairs.

**Sequestration of Yeast $\beta$ by Ga Subunits—**Receptor-bound heterotrimeric G proteins consist of GDP-Ga$\beta$$\gamma$ complexes. $\alpha_2$ subunits do not support coupling to G protein-coupled receptors in the absence of $\beta$ subunits (53). Since the G$_{\alpha_2}$, G$_{\alpha_2}$-gpa1, and Gpa1-G$_{16}$ chimeras in complex with PAR1 undergo high-affinity SFLLRN-dependent nucleotide exchange, this would indicate that the yeast $\beta$y dimer associates at least transiently with these Ga subunits. Indeed, if null mutants are made by deleting either the yeast STE4 or STE18 genes which encode the yeast $\beta$ and $\gamma$ subunits, respectively, one does not observe high affinity binding of $\alpha$-factor peptide to the Ste2 receptor (49). The intersubunit contacts between $\alpha_2$ and $\beta$ subunits are highly conserved between yeast and mammals (70% identity) and are well defined by x-ray structural analysis (25, 26). The major Ga switch I-II interface buries 1800 $\text{Å}^2$ of surface area with the side of the $\beta$ subunit (Fig. 6) and undergoes substantial conformational changes upon nucleotide exchange (25). A secondary interface between the top side of the $\beta$ subunit and the $\alpha$N helix of $\alpha_2$ provides an additional 900 $\text{Å}^2$ of binding surface.

To directly test for the ability of the hybrid mammalian Ga subunits to bind the yeast $\beta$y dimer we employed a $\beta$y sequestration assay. An essential function of the yeast Ga subunit, Gpa1, is to tightly sequester $\beta$y and thus prevent it from activating the MAP kinase scaffolding protein complex, Ste5-Ste20-Cdc24 (54, 55). Even minor leakage of free $\beta$y away from Ga results in activation of the MAP kinase cascade and mitotic arrest (34). The growth arrest phenotype is dependent on $\beta$y activation of the MAP kinase pathway which results in transcription of an integrated FUS1-HIS3 reporter gene (56). The growth arrest that would normally occur due to activation of the MAP kinase pathway is prevented by deletion of the
that exist in the N-terminal interface region. Together, these
labels on the Gα subunit are as follows: αN is the N-terminal α-helix involved in receptor and βγ coupling, αB domain is a unique Gpa1 domain of unknown function, SwII is part of the Go switch I-II interface that binds βγ and undergoes nucleotide-dependent conformational changes, the β6/α5 region is a C-terminal region involved in receptor coupling.

![Image](267x398 to 554x728)

**Fig. 6.** Domain-specific interactions between PAR1 and Go subunits. Composition of the hybrid Go subunits and their characteristics related to the heterotrimeric G protein structure (25). Labels on the Go subunit are as follows: αN is the N-terminal α-helix involved in receptor and βγ coupling, αB domain is a unique Gpa1 domain of unknown function, SwII is part of the Go switch I-II interface that binds βγ and undergoes nucleotide-dependent conformational changes, the β6/α5 region is a C-terminal region involved in receptor coupling.

PAR1 Thrombin Receptor-G Protein Interactions

FAR1 gene in KY187. Sequestration of βγ by a Gα subunit results in slow growth (long doubling time) due to failure to activate the MAP kinase pathway and transcription of the FUS1-HIS3 reporter gene. As shown in Table III, plasmid-expressed Gpa1 sequesters βγ and strongly retards growth (>15 h doubling time) in the absence of histidine. Addition of histidine to the media bypasses the necessity for the MAP kinase-dependent transcription of the FUS1-HIS3 reporter and restores growth (3.9-h doubling time). Conversely, in the absence of Gpa1 (vector alone), the βγ dimer freely activates the MAP kinase pathway, which results in the transcription of FUS1-HIS3. This causes a marked increase in growth (3.4-h doubling time) in histidine-deficient media. Expression of full-length Gi2 in KY187 results in little or no sequestration of yeast βγ by Gi2 in FAR1<sup>−</sup> yeast strains using a mitotic arrest assay.

In order to determine which domains of the Go subunit confer the ability to associate tightly with yeast βγ, the array of Gi hybrids were tested in the KY187 yeast strain. The Gα<sub>23</sub>-gpa1 chimera does not sequester βγ indicating that the N-terminal two-thirds of Gi2 does not provide sufficient positive interactions with yeast βγ. Conversely, the Gpa1-i2 and αN-i2 chimeras provide full sequestration of βγ (Table III). This had previously demonstrated very weak sequestration of yeast βγ by Gi2 in FAR1<sup>−</sup> yeast strains using a mitotic arrest assay.

<table>
<thead>
<tr>
<th>Table III: Sequestration of yeast βγ by plasmid-expressed Go subunits inhibits growth in histidine-deficient media</th>
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<tbody>
<tr>
<td><strong>Go chimera</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Gpa1</td>
</tr>
<tr>
<td>Vector alone</td>
</tr>
<tr>
<td>Gpa1-i2</td>
</tr>
<tr>
<td>G&lt;sub&gt;α&lt;/sub&gt;-gpa1</td>
</tr>
<tr>
<td>Gpa1-16</td>
</tr>
<tr>
<td>Gi2</td>
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<tr>
<td>αN-i2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Doubling time (dt) in hours from growth of gpa1 far1 his3 FUS1-HIS3 yeast KY187 carrying different Go-expressing plasmids in histidine-deficient media as measured by absorbance (A<sub>600</sub>; dt = 0.3(t2-t1)/logA2-logA1).

<sup>b</sup> Yeast culture growth over 2–3 days established only a lower limit of the doubling time.

<sup>c</sup> ND, not determined.

Data would imply that the forces holding together the heterotrimer are highly cooperative as suggested by x-ray structural analyses (25, 26).

**Dual Function of the Go Subunit αN Helix in Binding to βγ and in Coupling to Receptor**—The biochemical and genetic data presented here are the first evidence that the αN helix of the Go subunit plays a critical role in mediating proper coupling between G protein and receptor. The βγ-sequestration data demonstrate that binding determinants in the N-terminal αN helix are necessary but not sufficient for tight binding to the βγ dimer. Based on the x-ray structure of Gβγ (25), seven residues from the yeast Ste4 β subunit would comprise the binding surface for interaction with six residues from the bottom face of the αN helix of Go (Fig. 6). Three of the seven Ste4 N-terminal interface residues are non-conserved relative to mammalian β<sub>1</sub>
cognate Alanine scanning mutagenesis confirmed the importance of the PAR1, however, indicating that aies here, the having several non-conserved substitutions at surface-exposed residues of the α helix. Likewise, in our studies here, the α-i2 chimera bound tightly to PAR1 despite having several non-conserved substitutions at surface-exposed residues on the α helix. The α-i2 chimera did not couple to PAR1, however, indicating that α helix provides fine determinants of G protein-receptor signaling and is not simply docking the G protein to the receptor-membrane interface.

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