Signaling through Gα13 Switch Region I Is Essential for Protease-activated Receptor 1-mediated Human Platelet Shape Change, Aggregation, and Secretion*

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This study investigated the involvement of Gα13 switch region I (SRI) in protease-activated receptor 1 (PAR1)-mediated platelet function and signaling. To this end, myristoylated peptides representing the Gα13 SRI (Myr-G13SRIpep) and its random counterpart were evaluated for their effects on PAR1 activation. Initial studies demonstrated that Myr-G13SRIpep and Myr-G13SRIrandom-pep were equally taken up by human platelets and did not interfere with PAR1-ligand interaction. Subsequent experiments revealed that Myr-G13SRIpep specifically bound to platelet RhoA guanine nucleotide exchange factor (p115RhoGEF) and blocked PAR1-mediated RhoA activation in platelets and human embryonic kidney cells. These results suggest a direct interaction of Gα13 SRI with p115RhoGEF and a mechanism for Myr-G13SRIpep inhibition of RhoA activation.

Platelet function studies demonstrated that Myr-G13SRIpep specifically inhibited PAR1-stimulated shape change, aggregation, and secretion in a dose-dependent manner but did not inhibit platelet activation induced by either ADP or A23187. It was also found that Myr-G13SRIpep inhibited low dose, but not high dose, thrombin-induced aggregation. Additional experiments showed that PAR1-mediated calcium mobilization was partially blocked by Myr-G13SRIpep but not by the Rho kinase inhibitor Y-27632. Finally, Myr-G13SRIpep effectively inhibited PAR1-induced stress fiber formation and cell contraction in endothelial cells. Collectively, these results suggest the following: 1) interaction of Gα13 SRI with p115RhoGEF is required for G13-mediated RhoA activation in platelets; 2) signaling through the G13 pathway is critical for PAR1-mediated human platelet functional changes and low dose thrombin-induced aggregation; and 3) G13 signaling elicits calcium mobilization in human platelets through a Rho kinase-independent mechanism.

Thrombin and thrombin receptors in platelets play critical roles in heart attack, stroke, and thromboembolic processes (reviewed in Ref. 1). Consequently, investigating the mechanisms of thrombin receptor-mediated signaling pathways is of great importance to developing therapeutic approaches or anti-thrombotic agents (2) to treat certain cardiovascular diseases.

The first thrombin receptor was cloned and sequenced by Coughlin and co-workers in 1991 (3). It was found that this receptor is a member of the seven-transmembrane receptor class that signals through different G proteins. Thrombin acts by first cleaving the extracellular N-terminal domain of the receptor, and the new N terminus (SFLLRN) then binds to the second extracellular loop of the receptor protein to cause cellular activation (3). To date, four protease-activated receptors (PARs), i.e. PAR1, PAR2, PAR3, and PAR4, have been cloned, all of which except for PAR2 are identified as receptors for thrombin (4). Regarding platelets, there is a species-specific expression of the PAR subtypes. For example, PAR3 and PAR4 (but not PAR1) are expressed in mouse platelets. The functional significance of these receptors was demonstrated by the finding that knock-out of either PAR3 or PAR4 led to impaired thrombin-induced platelet activation (5). In contrast to mouse, human platelets possess PAR1 and PAR 4 but not PAR 3 receptors. Therefore, in this case, the combination of PAR1 and PAR4 signaling pathways is thought to account for a substantial portion of thrombin-mediated platelet activation (6).

As mentioned previously, PAR signaling pathways involve G protein-initiated effector activation. Furthermore, PARs are thought to couple to different G proteins depending upon the cell type. Our present understanding of PAR-G protein signaling in platelets is primarily based on the collective information obtained from experimental approaches in two separate species, i.e. mouse and human. Specifically, Offermanns et al. (7) evaluated the potential role of PAR-Gq signaling using Gq knock-out mice. The results using these mice demonstrated a complete inhibition of thrombin-induced platelet aggregation but not shape change (7). Further studies indicated that PARs may also be coupled to G12 (8), because thrombin caused a decrease in cAMP levels in the wild-type as well as in the Gq null mouse platelets. As an extension of this work, the effects of a conditional G13 knock-out on mouse platelet function were also evaluated (9). It was found that the G13 null platelets exhibit...
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EXPERIMENTAL PROCEDURES

Reagents—Myristoylated (Myr) Gα₁₃ SRI peptide (Myr-LLARRPDKGHIHEY, Myr-G₁₃SRI_pep) and Myr-Gα₁₃SRI random peptide (Myr-LIRYPHRATKEG, Myr-G₁₃SRIRandom_pep) were synthesized by GenScript Corp. (Piscataway, NJ) or the Research Resource Center, University of Illinois, Chicago; biotinylated (Bio) Gα₁₃ SRI peptide (Bio-LLARRPDKGHIHEY, Bio-G₁₃SRI_pep), Bio-Gα₁₃ SRI random peptide (Bio-LIRYPHRATKEG, Bio-G₁₃SRIRandom_pep), Myr-Bio-Gα₁₃ SRI peptide (Myr-Bio-LLARRPDKGHIHEY, Myr-Bio-G₁₃SRIpep), Myr-Bio-Gα₁₃SRI random peptide (Myr-Bio-LIRYPHRATKEG, Myr-Bio-G₁₃SRIRandom_pep), thrombin receptor agonist peptide (SFLLRNPNKYEFP, TRAP1), and PAR4-activating peptide (AYPGKF) were synthesized by the Research Resource Center, University of Illinois, Chicago. Luciferin/luciferase reagent (Chrono-lume) was purchased from Chrono-Log (Havertown, PA); HRP-conjugated goat anti-biotin was from Vector (Burlingame, CA); HRP-conjugated goat anti-rabbit IgG (H + L) was from Bio-Rad; human embryonic kidney (HEK) cells and human live microvascular endothelial cells were purchased from the American Type Culture Collection (ATCC). Polyvinylidene difluoride membranes were from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence substrate, biotinylation reagent EZ-Link Biotin-LC-PEO-Amine, and the BCA protein assay kit were from Pierce. Bovine serum albumin (BSA), tetramethylbenzidine (TMB), apyrase (grade VII) and Y-27632 were from Sigma. Protein kinase C inhibitor Ro 31-8220 was from Cayman (Ann Arbor, MI). Nunc-ImmuNoTM plates were from Fisher. ³H-TRAP1 (specific activity 19.55Ci/mM) was custom- labeled by Amersham Biosciences. Fura-2AM was from Molecular Probes (Eugene, OR). Human platelet-rich plasma (PRP, anticoagulated with acid/ citrate/dextrose, 2–3 × 10⁵ platelets/ml) were purchased from Life Source Blood Services (Glenview, IL), and outdated platelets were kindly provided by the Hospital Blood Bank, University of Illinois, Chicago.

Platelet Membrane Preparation and Solubilization—Solubilized platelet membranes were prepared as described previously (20). Briefly, outdated human platelets were incubated with 3 mM aspirin for 45 min and pelleted by centrifugation at 1,600 × g for 20 min. The platelet pellets were washed with buffer A (25 mM Tris-HCl, 5 mM MgCl₂, pH 7.4) containing PGI₂ (40 nM), sonicated on ice, and centrifuged at 1,600 × g for 5 min. The supernatant was placed into centrifuge tubes, and the pellets were again resuspended, sonicated, and centrifuged. The supernatants were then combined and centrifuged at 100,000 × g for 30 min. The pellet was resuspended in buffer A plus 10 mM CHAPS, homogenized, and centrifuged again at 100,000 × g for 30 min. The resulting membrane pellet was resuspended in buffer A to yield a final protein concentration of 2–4 mg/ml.

Other reports have provided indirect evidence linking the human G₁₂/₁₃ signaling pathway to PAR1-mediated platelet function (13–16). Thus, it was found that selective activation of PAR1 with the peptide YFLLRNP resulted in platelet shape change (13) that could be blocked by the Rho kinase inhibitor Y-27632 (14, 15). Because RhoA activation has been linked previously to G₁₃ signaling (8, 17), these results led the authors to suggest that this shape change response is mediated through G₁₃, and possibly G₁₂, signaling involvement of intracellular calcium. Taken together, these data indicate that signaling of G₁₃ through SRI is critical for PAR1-induced cellular activation.

EXPERIMENTAL PROCEDURES

Reagents—Myristoylated (Myr) Gα₁₃ SRI peptide (Myr-LLARRPDKGHIHEY, Myr-G₁₃SRI_pep) and Myr-Gα₁₃SRI random peptide (Myr-LIRYPHRATKEG, Myr-G₁₃SRIRandom_pep) were synthesized by GenScript Corp. (Piscataway, NJ) or the Research Resource Center, University of Illinois, Chicago; biotinylated (Bio) Gα₁₃ SRI peptide (Bio-LLARRPDKGHIHEY, Bio-G₁₃SRI_pep), Bio-Gα₁₃ SRI random peptide (Bio-LIRYPHRATKEG, Bio-G₁₃SRIRandom_pep), Myr-Bio-Gα₁₃ SRI peptide (Myr-Bio-LLARRPDKGHIHEY, Myr-Bio-G₁₃SRIpep), Myr-Bio-Gα₁₃SRI random peptide (Myr-Bio-LIRYPHRATKEG, Myr-Bio-G₁₃SRIRandom_pep), thrombin receptor agonist peptide (SFLLRNPNKYEFP, TRAP1), and PAR4-activating peptide (AYPGKF) were synthesized by the Research Resource Center, University of Illinois, Chicago. Luciferin/luciferase reagent (Chrono-lume) was purchased from Chrono-Log (Havertown, PA); HRP-conjugated goat anti-biotin was from Vector Laboratories (Burlingame, CA); HRP-conjugated goat anti-rabbit IgG (H + L) was from Bio-Rad; human embryonic kidney (HEK) cells and human live microvascular endothelial cells were purchased from the American Type Culture Collection (ATCC). Polyvinylidene difluoride membranes were from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence substrate, biotinylation reagent EZ-Link Biotin-LC-PEO-Amine, and the BCA protein assay kit were from Pierce. Bovine serum albumin (BSA), tetramethylbenzidine (TMB), apyrase (grade VII) and Y-27632 were from Sigma. Protein kinase C inhibitor Ro 31-8220 was from Cayman (Ann Arbor, MI). Nunc-ImmuNoTM plates were from Fisher. ³H-TRAP1 (specific activity 19.55Ci/mM) was custom- labeled by Amersham Biosciences. Fura-2AM was from Molecular Probes (Eugene, OR). Human platelet-rich plasma (PRP, anticoagulated with acid/citrate/dextrose, 2–3 × 10⁵ platelets/ml) were purchased from Life Source Blood Services (Glenview, IL), and outdated platelets were kindly provided by the Hospital Blood Bank, University of Illinois, Chicago.

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Purification of Recombinant p115—Full-length p115RhoGEF cDNA was ligated into BamHI, Xhol sites of pFastBacHTc vector with hexahistidine tag at the N terminus. Baculovirus expressing His$_6$-p115 was generated, and SF9 cells were infected with the virus for 48 h. Recombinant p115 protein was purified from the cytosolic fractions of infected cells using nickel-nitrilotriacetic acid affinity column with the yield of 0.5 mg from 500 ml of SF9 cell culture. The protein was 90% pure by SDS-PAGE visualization.

Dot Blot for Myristoylated Peptides—Human PRP was incubated with 100 |M| biotinylated Myr peptides or free biotinylating reagent (as a secondary control) for 5 min. The platelets were then washed three times by centrifugation in Tyrode’s buffer containing PGI$_2$ (40 nM), disrupted with lysis buffer (50 mM Tris-HCl containing 100 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 10% glycerol, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol, 50 |g|/ml phenylmethysulfonyl fluoride, 10 |g|/ml each of leupeptin and pepstatin, and 0.2% Nonidet P-40, pH 7.5), and sonicated on ice (three 45-s bursts with 15-s pauses). The platelet samples were next loaded on methanol-pretested polyvinylidene difluoride membranes, blocked by 5% nonfat dry milk, and incubated with HRP-conjugated goat anti-biotin Ab at room temperature for 1 h. After incubation, the membranes were washed three times with Tyrode’s buffer and subsequently incubated with HRP substrates. The film was then developed and fixed.

Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometric Analysis (MALDI-TOFMS)—MALDI-TOFMS analysis was performed as described previously (21). Specifically, human PRP was incubated with 500 |g|/ml Myr-G13SRIpep or Myr-G13SRIRandom-pep for 5 min at room temperature. Following incubation, the platelets were washed three times with Tyrode’s buffer containing PGI$_2$ (40 nm) and disrupted with lysis buffer and sonicated as described above. The platelet samples were then mixed 1:1 with the matrix solution (10 mg of cyanogen-4-hydroxycinnamic acid in 1 ml of aqueous solution of 50% acetonitrile containing 0.1% trifluoroacetic acid). Finally, the sample aliquots were spotted onto a MALDITOF target and analyzed at 1,764 daltons using a Voyager-DE PRO mass spectrometer (Applied Biosystems) equipped with a 337 nm pulsed nitrogen laser.

$^3$H-TRAP1 Binding to Intact Human Platelets—To ensure that the association of $^3$H-TRAP1 with platelets was not because of uptake of the ligand, displacement studies were employed to measure the extent of the specific binding. In these studies, PRP was initially incubated with 500 |g|/ml Myr-G13SRIpep or Myr-G13SRIRandom-pep for 5 min at room temperature. At this point, 40 nm $^3$H-TRAP1 was added, and the reaction mixture was incubated at 4°C for 10 min. The reaction was then supplemented with 40 |g| unlabeled TRAP1 (to determine specific binding) and incubated for an additional 10 min. Aliquots (1 ml) of the PRP were immediately layered over a silicon oil mixture (75; 5|l|; 81% Dow 550; 19% Dow 200) in a centrifuge tube and centrifuged at 7,000 $\times$ g for 1 min to separate the platelets from their plasma (22, 23). The supernatant was quickly removed by aspiration, and the tube tips containing the platelet pellets were cut and transferred to scintillation vials. One ml of 0.3 N NaOH was added to solubilize the platelets, and 100 |l| of 3 N HCl were added to neutralize the reaction mixture. Finally, 10 ml of scintillation fluid were added, and the solution was counted on a Beckman LS 6500 liquid scintillation counter. Under these conditions, specific binding of $^3$H-TRAP1 was 33%.

Pulldown Assay for GTP-Rho—RhoA activation was determined as described previously with slight modifications (24). Briefly, 400 |l| of PRP were incubated with 500 |g|/ml Myr-G13SRIpep, 500 |g|/ml Myr-G13SRIRandom-pep, or Me$_2$SO vehicle at room temperature for 5 min and then treated with 25 |g|/ml PAR1 agonist (TRAP1) for 1 min. One volume of cold lysis buffer was added, and the samples were incubated on ice for 10 min. The platelet lysates were then centrifuged at 15,000 $\times$ g for 1 min, and the supernatant was incubated (4°C for 1 h) with 20 |g| of glutathione S-transferase-Rho binding domain fusion protein-conjugated beads. The beads were washed three times with lysis buffer and subjected to SDS-PAGE. Activated RhoA was detected by Western blot analysis using a polyclonal antibody against RhoA. In separate experiments, 400 |l| of HEK cells were incubated with 500 |g|/ml Myr-G13SRIpep, 500 |g|/ml Myr-G13SRIRandom-pep, or Me$_2$SO vehicle at room temperature for 15 min and then treated with 50 |g| TRAP1 for 1 min. After centrifugation, 400 |l| of cold lysis buffer was added, and the sample was sonicated on ice. The cell lysates were then centrifuged at 15,000 $\times$ g for 1 min, and the supernatant was subjected to the same procedure as described above.

Measurement of p115 RhoGEF Binding to G13SRIpep—The design of this experiment was to coat Nunc-Immuno plate wells with G13SRIpep or G13SRIRandom-pep and to measure whether either peptide could capture p115RhoGEF from solubilized human platelets using a sandwich ELISA. However, to establish that equal amounts of G13SRIpep and G13SRIRandom-pep were immobilized to the wells, the following experiment was first conducted. In brief, the wells were coated with various concentrations (0.1 nm to 400 |g|) of biotinylated G13SRIpep or biotinylated G13SRIRandom-pep at 4°C overnight. The wells were then washed three times with TBS buffer containing 0.03% Tween 20 and blocked with 3% BSA in TBS-Tween buffer. Following incubation at room temperature for 1 h with avidin-HRP, the wells were washed seven times with TBS-Tween buffer, and the TMB substrates were added. When the appropriate color appeared, 50 |l| of 1 N HCl were added to stop the reaction, and the absorbance (A) at 450 nm was measured in each sample. A comparison of the A$_{450}$ values revealed that 0.23 |g|/ml G13SRIpep and 12.5 |g|/ml G13SRIRandom-pep resulted in equal coating of the wells (Fig. 5A).

In the sandwich ELISA experiments, the Nunc-Immuno plate wells were equally coated with either G13SRIpep or G13SRIRandom-pep and blocked as outlined above. After washing three times with TBS-Tween buffer, 100 |g| of solubilized platelet membrane protein (20) were added, and the samples were incubated at room temperature for 2 h. The wells were then supplemented with p115RhoGEF Ab and incubated at room temperature for an additional 1 h. After three washes with TBS-Tween buffer, the HRP-linked secondary Ab was added, and the reaction was incubated at room temperature for an additional 1 h. Finally, TMB substrates were added, and the A$_{450}$ values were measured.
In alternative ELISA experiments, the Nunc-Immuno plate wells were coated with 100 µg/ml recombinant human p115RhoGEF and blocked with 3% BSA. The wells were then supplemented with Bio-G_{13}SRI_{pep} or Bio-G_{13}SRI_{random-pep}. After incubation at 37 °C for 4 h, the avidin-HRP was added, and the A_{405} values were measured as described above.

Platelet Shape Change—Human PRP was incubated with Me_{2}SO (vehicle control), Myr-G_{13}SRI_{pep} or Myr-G_{13}SRI_{random-pep} for 5 min, followed by addition of 3 µM EGTA and 10 µM indomethacin. After 2 min of incubation, shape change was induced by adding different agonists and measured by the turbidimetric method (23) using a model 400 aggregometer (Chrono-Log, Havertown, PA).

Platelet Aggregation in PRP—Human PRP was incubated with Me_{2}SO vehicle, Myr-G_{13}SRI_{pep} or Myr-G_{13}SRI_{random-pep} at room temperature for 5 min and 10 µM indomethacin for 2 min. Platelet aggregation was induced by adding different agonists, and measured by the turbidimetric method (23) using a model 400 aggregometer (Chrono-Log).

Platelet Aggregation in Resuspended Platelets—Human PRP was incubated with 500 µM Myr-G_{13}SRI_{pep} or Myr-G_{13}SRI_{random-pep} for 5 min. Following the addition of 40 nM PGI_{2}, the plasma was removed by centrifugation at 500 × g for 3 min. Platelets were resuspended in Tyrode’s buffer (supplemented with 1 mM calcium and 0.38% BSA), and the platelet count was adjusted to 3 × 10^{10} platelets/ml. Following addition of 10 µM indomethacin and 0.05 units/ml apyrase, thrombin (0.1 or 0.2 units/ml) was added, and platelet aggregation was measured.

Platelet Dense Granule Secretion—Human PRP was preincubated with Me_{2}SO vehicle, 75–250 µM Myr-G_{13}SRI_{pep} or 250 µM Myr-G_{13}SRI_{random-pep} and incubated with luciferin/luciferase for 3 min before stimulation. Platelet dense granule secretion was induced by adding 25 µM TRAP1, and secreted ATP was measured with a Chrono-Log lumi-aggregometer.

Platelet Calcium Mobilization—Human PRP was incubated with Fura-2 and 500 µM Myr-G_{13}SRI_{pep} (or 500 µM Myr-G_{13}SRI_{random-pep}) for 30 min. The platelets were then supplemented with 10 µM indomethacin, 0.05 units/ml apyrase and sedimented by centrifugation at 500 × g for 3 min. Following resuspension in Tyrode’s buffer (supplemented with 1 mM calcium, 0.38% BSA, 10 µM indomethacin, and 0.05 units/ml apyrase), platelet calcium mobilization was induced with 25 µM TRAP1 or 10 µM ADP. In the experiments with Y-27632, the platelets were loaded with Fura-2 as described above. Platelets were then incubated with vehicle or 30 µM Y-27632 for 3 min, and calcium mobilization was induced with 25 µM TRAP1. The samples were excited at 340/380 nm, and the fluorescent emissions were detected at 510 nm using a PTI spectrofluorometer.

Stress Fiber Formation and Cell Contraction—Human live microvascular endothelial cells were seeded on coverslips and starved for 24 h after the cells became 100% confluent. The cells were then preincubated with 500 µM Myr-G_{13}SRI_{random-pep}, Myr-G_{13}SRI_{pep} or vehicle (in 6% BSA Hanks’ buffer) for 30 min and stimulated with 50 µM TRAP1 or vehicle for 15 min. After fixation with 3.7% formaldehyde and permeabilization with 0.1% Triton X-100, the cells were stained with Alex Fluor 488 phalloidin/4’,6-diamidino-2-phenylindole. Finally, the cells were washed and mounted, and the images were obtained using a confocal microscope (Zeiss LSM 510).

Statistical Analysis—Data were analyzed with GraphPad PRISM statistical software (San Diego, CA). Statistical significance is defined as p < 0.05 or p < 0.0001, as indicated.

RESULTS

Characterization of Myr-G_{13}SRI_{pep} Uptake by Intact Human Platelets—Our previous studies showed that a peptide representing G_{13}SRI can be useful to study G_{13} signaling (24). To further explore G_{13} signal transduction in intact human platelets, myristoylated peptides representing the G_{13}SRI (Myr-G_{13}SRI_{pep}) and its random counterpart (Myr-G_{13}SRI_{random-pep}) were synthesized. Although earlier reports have demonstrated that the myristoylation of peptides can serve as an effective means to increase their permeability (25, 26), it was first necessary to establish that the Myr-G_{13}SRI peptides can indeed be taken up by intact platelets. In the initial experiments, the biotinylated derivatives of Myr-G_{13}SRI_{pep} and Myr-G_{13}SRI_{random-pep} were employed. The uptake of the biotinylated peptides by intact platelets was then evaluated by dot blot analysis. As seen in Fig. 1A, the platelets that were incubated with either biotinylated Myr-G_{13}SRI_{pep} or biotinylated Myr-G_{13}SRI_{random-pep} revealed a strong positive stain relative to the platelets incubated with the biotinylation reagent alone. These results indicate that both peptides are taken up by intact platelets and that the degree of uptake is comparable for Myr-G_{13}SRI_{pep} and Myr-G_{13}SRI_{random-pep}.

This notion was further confirmed by separate experiments using the nonbiotinylated myristoylated peptides. In these studies the uptake of each peptide was evaluated using MALDI-TOFMS, as described previously (21). It can be seen that the 1,764-dalton peptides were present in both Myr-G_{13}SRI_{pep}-treated (Fig. 1B) and Myr-G_{13}SRI_{random-pep}-treated platelet samples (Fig. 1C). Collectively, the above results establish that both Myr-G_{13}SRI_{pep} and G_{13}SRI_{random-pep} are taken up by intact human platelets.

Effect of Myr-G_{13}SRI_{pep} on ³H-TRAP1 Binding to Human Platelets—To examine whether Myr-G_{13}SRI_{pep} affects PAR1-ligand interaction, ³H-TRAP1 binding experiments were conducted in intact human platelets. The results demonstrated (Fig. 2A) that there was no difference in ³H-TRAP1 specific binding between the Myr-G_{13}SRI_{pep}-treated and the Myr-G_{13}SRI_{random-pep}-treated platelets. This result therefore provides evidence that the inhibition of RhoA activation by Myr-G_{13}SRI_{pep} is not through inhibition of ligand binding to PAR1.

Effects of Myr-G_{13}SRI_{pep} on PAR1-induced RhoA Activation in Intact Human Platelets and HEK Cells—The next experiments examined the effects of Myr-G_{13}SRI_{pep} and Myr-G_{13}SRI_{random-pep} on PAR1-mediated RhoA stimulation in intact platelets and HEK cells. RhoA activation was determined as described previously with slight modifications (24). As can be seen in Fig. 2B and C, treatment of human platelets or HEK cells with Myr-G_{13}SRI_{pep} blocked TRAP1-induced RhoA activation, whereas treatment with Myr-G_{13}SRI_{random-pep} was without apparent effect. These results demonstrate that the SRI amino acid sequence of G_{13} is an effective peptide inhibitor of PAR1 signaling through the G_{13}-RhoA pathway.
Measurement of p115 RhoGEF Binding to G_{13}SRI—Previous studies (27) using recombinant p115RhoGEF and G_{13} have suggested that G_{13}SRI may serve as a binding domain for p115RhoGEF. To further investigate this mechanism in human platelets, a sandwich ELISA was performed to measure possible binding interactions between these signaling molecules. In this experiment Nunc-Immuno plate wells were first coated with G_{13}SRIpep or G_{13}SRIRandom-pep. The ability of either peptide to capture p115RhoGEF from solubilized human platelets was then measured. However, to ensure proper interpretation of the results, it was first necessary to establish that equal amounts of G_{13}SRIpep and G_{13}SRIRandom-pep were immobilized to the plate wells. This was accomplished by coating the wells with various concentrations (0.1 nM to 400 μM) of biotinylated G_{13}SRIpep or biotinylated G_{13}SRIRandom-pep. Following incubation with avidin-HRP, the substrates were added, and the A_{450} values were measured in each sample. It was found that 0.23 μM G_{13}SRIpep and 12.5 μM G_{13}SRIRandom-pep resulted in equal coating of the wells (Fig. 3A).

In the next experiments, the wells were equally coated and subjected to a sandwich ELISA (see under “Experimental Procedures”). The data are representative of results obtained using platelets from three different donors.  B, Myr-G_{13}SRIpep inhibits TRAP1-induced platelet RhoA activation. Human PRP was incubated with 500 μM Myr-G_{13}SRIpep or vehicle. The platelets were then treated with 25 μM TRAP1 and subjected to a GTP-Rho pulldown assay (see details under “Experimental Procedures”). The RhoA Western blot is representative of results obtained using platelets from six different donors. C, Myr-G_{13}SRIpep inhibits TRAP1-induced HEK cell RhoA activation. HEK cells were incubated with 500 μM Myr-G_{13}SRIpep, Myr-G_{13}SRIRandom-pep, or vehicle. The cells were then treated with 50 μM TRAP1 and subjected to the GTP-Rho pulldown assay. The RhoA Western blot is representative of results obtained from three independent experiments.
p115RhoGEF bound significantly more G13SRIpep than the random peptide. Taken together, these results demonstrate that the G13SRI amino sequence binds to both endogenous platelet p115RhoGEF, as well as to recombinant p115RhoGEF.

Effects of Myr-G13SRIpep on PAR1-mediated Human Platelet Shape Change—Earlier studies have suggested that platelet shape change proceeds at least in part through G13 signaling (8, 9, 17). The next experiments determined the involvement of this signaling pathway in the PAR1-mediated human platelet shape change response. It was found that Myr-G13SRIpep inhibited TRAP1-induced platelet shape change in a dose-dependent manner (Fig. 4, A and B). On the other hand, a similar inhibition was not observed with either the nonmyristoylated G13SRIpep or the Myr-G13SRIRandom-pep (Fig. 4C). To determine the specificity of the Myr-G13SRIpep inhibition, parallel experiments were conducted using separate stimulating agents that induce shape change through mechanisms independent of G13 signaling, i.e. ADP and the ionophore A23187. The results demonstrated, however, that 500 μM Myr-G13SRIpep produced no apparent inhibition of platelet shape change in response to either ADP (10 μM) or A23187 (2 μM) (Fig. 4D and E).

Effects of Myr-G13SRIpep on PAR1-mediated Human Platelet Aggregation—Although previous studies have suggested that G13 can participate in human platelet aggregation, the contribution of this signaling pathway to the overall aggregation response is presently unknown. The next series of experiments investigated the requirement for G13 signaling in PAR1-mediated human platelet aggregation. As can be seen in Fig. 5, A and B, Myr-G13SRIpep dose-dependently inhibited TRAP1-induced platelet aggregation, whereas nonmyristoylated G13SRIpep and Myr-G13SRIRandom-pep revealed no inhibitory effects (Fig. 5C). Again, to examine the specificity of the Myr-G13SRIpep inhibition, ADP and A23187 were tested in parallel experiments. It was found that 500 μM Myr-G13SRIpep did not affect ADP (Fig. 5D) or A23187-induced platelet aggregation (Fig. 5E).

Effects of Myr-G13SRIpep on PAR1-mediated Human Platelet Aggregation in the Absence of Platelet Dense Granule Secretion—Because the overall aggregation response to PAR1 activation actually represents a composite of contributions from primary aggregation and aggregation derived from secreted products, e.g. ADP, the observed inhibition of aggregation (Fig. 5) may in part derive from inhibition of secretion. To specifically test the involvement of G13 signaling in the process of primary aggregation, experiments were conducted using the protein kinase C inhibitor Ro 31-8220, which is known to block platelet dense granule release (28). It can be seen that treatment with 10 μM Ro 31-8220 effectively inhibited the platelet secretion response, as measured by ATP release (Fig. 6A). It can also be seen that in the absence of dense granule secretion, Myr-G13SRIpep still produced substantial inhibition of PAR1-mediated platelet aggregation (Fig. 6B). These results therefore demonstrate that G13SRIpep has the capacity to block PAR1-mediated primary

FIGURE 3. G13SRIpep binds to human platelet p115 RhoGEF. A, Nunc-Immuno plate wells were coated with various concentrations of biotinylated G13SRIpep or biotinylated G13SRIrandom-pep. Avidin-HRP was added and detected (see details under "Experimental Procedures"). As can be seen, 0.23 μM biotinylated G13SRIpep and 12.5 μM biotinylated G13SRIrandom-pep resulted in equal coating of the wells. B, Nunc-Immuno plate wells were equally coated with G13SRIpep and G13SRIrandom-pep. Platelet membrane protein was added, and sandwich ELISA was performed (see details under "Experimental Procedures"). The A450 values reflect the relative amount of platelet p115 RhoGEF captured by G13SRIpep and G13SRIrandom-pep (*, p < 0.0001). The data are obtained from 10 independent experiments. C, Nunc-Immuno plate wells were coated with recombinant p115 RhoGEF, and then supplemented with Bio-G13SRIpep or Bio-G13SRIrandom-pep. Avidin-HRP and TMB substrates were subsequently added to detect the biotinylated peptides captured by recombinant p115 RhoGEF (*, p < 0.002). The data are obtained from seven independent experiments.
aggregation and suggest a significant role for Gα13 signaling in this aggregation response.

Effects of Myr-G13SRIpep on PAR1-mediated Human Platelet Dense Granule Secretion—Because G13 signaling is involved in RhoA activation and cytoskeletal rearrangement (8), this pathway may also contribute to platelet secretion. To investigate this possibility, the effects of Myr-G13SRIpep on PAR1-induced dense granule release were measured. It was found that Myr-G13SRIpep dose-dependently inhibited TRAP1-stimulated ATP secretion (Fig. 7, A and B), whereas Myr-G13SRIRandom-pep revealed no inhibitory effects (Fig. 7B). Taken together, the above studies provide evidence that G13 signaling is essential for PAR1-mediated human platelet shape change, aggregation, and dense granule secretion. These results further suggest a critical role for Gα13 SRI in these separate platelet functional responses.

Effects of Myr-G13SRIpep on PAR1-mediated Human Platelet Calcium Mobilization—Calcium mobilization has been linked to all the major platelet functional changes including shape change, aggregation, and secretion. In addition, Gq signaling through PLCβ and inositol 1,4,5-trisphosphate is commonly thought to be responsible for the initiation of this calcium mobilization process (29–35). Although it has been speculated that G13 activation may also lead to intraplatelet calcium release (14, 36), no direct evidence has thus far been provided to establish this alternate calcium signaling mechanism. To explore this issue, the effects of Myr-G13SRIpep on PAR1-induced human platelet calcium mobilization were tested. It was found that relative to Myr-G13SRIRandom-pep, Myr-G13SRIpep inhibited TRAP1-mediated platelet calcium mobilization by ~30% (Fig. 8, A–C). In contrast, treatment of platelets with the Rho kinase inhibitor Y-27632 did not reveal inhibition of the calcium mobilization response (Fig. 8, D–F). To test the effects of Myr-G13SRIpep on ADP-induced calcium mobilization, separate experiments were conducted. As can be seen in Fig. 8, G–I, Myr-G13SRIpep did not interfere with this calcium mobilization response. Collectively, these results indicate that Gα13 signaling has the capacity to stimulate human platelet calcium mobilization through a Rho kinase-independent mechanism.

Effects of Myr-G13SRIpep on Thrombin-mediated Human Platelet Aggregation—The following experiments examined the relative contribution of PAR1-G13 signaling to the overall thrombin-induced platelet aggregation response. It was found that Myr-G13SRIpep substantially inhibited platelet aggregation induced by 0.1 unit/ml thrombin, indicating a role for G13 signaling at this thrombin dose (Fig. 9A). On the other hand, when the thrombin concentration was raised to 0.2 units/ml, Myr-
we evaluated the effects of Myr-\(G_{13}\)SRI_{pep} on TRAP1-induced endothelial cell stress fiber formation and cell contraction. Specifically, human live microvascular endothelial cells were preincubated with 500 \(\mu M\) Myr-\(G_{13}\)SRI_{random-pep} or vehicle for 30 min prior to addition of 50 \(\mu M\) TRAP1 or vehicle. After fixation and permeabilization, the cells were stained, washed, and mounted. It can be seen that PAR1 activation with TRAP1 caused both stress fiber formation (Fig. 10A) and cell contraction (Fig 10B). It can also be seen that treatment of the cells with Myr-\(G_{13}\)SRI_{pep} prevented these \(G_{13}\)-mediated functional changes. On the other hand, treatment with Myr-\(G_{13}\)SRI_{random-pep} (500 \(\mu M\)) was ineffective in preventing endothelial cell activation.

**DISCUSSION**

Although it is known that platelets possess multiple G protein signaling pathways that contribute to the different platelet functional responses, the relative participation of these individual pathways in platelet shape change, aggregation, and secretion is not well characterized. To a large extent this is because of the lack of suitable reagents that selectively interfere with specific G protein signaling events and that can be applied to the study of intact human platelets. With the exception of receptor-derived peptides (40–42), and G protein \(\alpha\) subunit C-terminal peptides (40, 43, 44) that modulate receptor-G protein coupling, the field has for the most part been limited to agents that interfere with different downstream kinases or other downstream effectors. However, the separate G protein pathways share many of these downstream targets, and consequently it has been difficult to assign a specific platelet function to a certain G protein. To address this issue, it was reasoned that more direct information about specific G protein involvement in cellular signaling might be obtained by interfering with the initial G protein signal transduction event, rather than by interfering with the secondary downstream consequences of this transduction process. Based on this consideration, experiments were undertaken to design and test specific inhibitors of G protein signaling at the G protein level itself. The
SRI of the Ga subunit was selected as a possible region of interest, because previous studies have indicated the importance of this Ga domain for the initial transduction of G protein signal-

ing (24, 27, 45). Furthermore, the G13 pathway was chosen for these experiments because it remains the least studied G protein in human platelets, and because new information could be obtained regarding its participation in the platelet activation process. In addition, G13 versus G12 signaling was selected for study, because the G12 pathway does not seem to play an important role in platelet activation (9, 36, 46). To this end, we have designed a permeable peptide (Myr-G13SRIpep) representing the conformationally sensitive SRI of the Gα13 subunit, and we have examined its effects on human platelet signaling and function through PAR1, which is known to couple to Gα13. In the initial experiments, dot blot and MALDI-TOFMS analysis provided evidence that both Myr-G13SRIpep and Myr-G13SRIrandom-pep are taken up by intact human platelets. In addition, separate studies demonstrated that the non-MyrG13SRIpep has no apparent effect on platelet function. Taken together, these results therefore indicate that uptake of G13SRIpep into platelets is required for its effects on G13 signaling.

The next series of experiments investigated the downstream events involved in Gα13 activation. It was found that Myr-G13SRIpep produced a substantial inhibition of PAR1-mediated RhoA activation, suggesting that the SRI of Gα13 participates in this activation process. Furthermore, one likely candidate for the site of this inhibition is p115RhoGEF, because previous results have indicated that activated Gα13 SRI transduces its signal (at least in part) through interaction with this guanine nucleotide exchange factor. This possibility was investigated using sandwich ELISA. It was found that Myr-G13SRIpep was indeed capable of directly binding to p115RhoGEF, suggesting that the mechanism by which Myr-G13SRIpep inhibits PAR1-induced RhoA signaling is by blocking the interaction between Gα13 SRI and p115RhoGEF. These results also provide insight into the mechanism of RhoA activation by PAR1. Specifically, it has been suggested that RhoA can be stimulated not only by Gα13 signaling but also by Gq signaling (10, 47). Furthermore, PAR1 is known to couple to both Gα13 and Gαq (7, 9, 10). Thus, the overall activation of RhoA by PAR1 would be expected to be a composite of activation through both signaling pathways. However, because Myr-G13SRIpep dramatically inhibits TRAP1-induced RhoA activation, it can be concluded that PAR1-mediated RhoA signaling primarily proceeds through the Gα13 pathway.

The next series of experiments evaluated the involvement of Gα13 signaling in human platelet shape change mediated by PAR1. Because PAR1 has the capacity to couple to both Gα13 and Gαq, it has been difficult to conclusively establish the relative contribution of each signaling pathway to the shape change response. Furthermore, the use of the Rho kinase inhibitor
Y-27632 cannot be employed to distinguish between these two possibilities, because as mentioned above, RhoA may be activated by both Gq/H925113 and Gq/q signaling (10, 47). The availability of Myr-G13SRIpep therefore provided a unique opportunity to study the separate signaling pathways associated with the platelet shape change response. It was found that Myr-G13SRIpep dose-dependently inhibited PAR1-induced shape change but not shape change induced by reagents that cause platelet activation through Gq-independent mechanisms, i.e. ADP or A23187. Because ADP-induced shape change is a Gq-mediated event (10), the inability of Myr-G13SRIpep to block this ADP response has two important implications. First, it establishes that Myr-G13SRIpep differentiates between G13 and Gq signaling. Second, it indicates that although both Gq and G13 can contribute to the shape change response (17), PAR1-induced human platelet shape change heavily depends on G13 SRI signaling.

The present results also provide new information concerning the role of G13 signaling in PAR1-mediated human platelet aggregation. In this connection, previous studies have provided indirect evidence that the G13 pathway can contribute to the human platelet aggregation response. Specifically, when low doses of agonists were used in an attempt to select for G12/13 and Gi signaling, it was found that co-stimulation of these pathways resulted in a platelet aggregation response, as well as \\n
\[ G_{13} \text{ signaling} \]

FIGURE 8. PAR1-induced platelet calcium mobilization involves G13 SRI signaling. A–C, human PRP was incubated with Fura-2 and 500 \( \mu \)M Myr-G13SRIpep or Myr-G13SRIrandom-pep. Platelets were then pretreated with 10 \( \mu \)M indomethacin, 0.05 units/ml apyrase, and platelet calcium mobilization was induced by adding 25 \( \mu \)M TRAP1 (*, \( p < 0.02 \)). The calcium mobilization curves are representative of results obtained using platelets from five different donors. D–F, human PRP was incubated with Fura-2, followed by treatment with vehicle or 30 \( \mu \)M Y-27632. Platelet calcium mobilization was stimulated with 25 \( \mu \)M TRAP1 (p > 0.5). The calcium mobilization curves are representative of results obtained using platelets from five different donors.

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FIGURE 9. G13 SRI is required for low dose thrombin-mediated human platelet aggregation. A and B, human PRP was incubated with 500 \( \mu \)M Myr-G13SRIpep or Myr-G13SRIrandom-pep. The plasma was removed, and the platelets were resuspended in Tyrode's buffer (see details under “Experimental Procedures”). Platelet aggregation was induced by 0.1 unit/ml (A) or 0.2 units/ml (B) thrombin. The platelet aggregation curves are representative of results obtained using platelets from three different donors.
capacity to participate in PAR1-induced primary aggregation but also that its signaling is essential for this aggregation process. Although the mechanism of PAR1-G13SRI-mediated aggregation is unknown, it presumably does not involve the RhoA pathway, because previous results have demonstrated that ADP-riboseylation of RhoA has no apparent effect on TRAP1-induced human platelet aggregation (48). Consequently, our results support the previous suggestion (14, 18) that Gα13 has additional transduction pathways in platelets that are independent of RhoA. Indeed, the present results provide the first evidence that such an alternative pathway involves calcium signaling. Thus, it was found that interruption of PAR1-G13SRI signaling with Myr-G13SRI-pep caused a reduction in the platelet calcium mobilization response. Interestingly, a similar inhibition of calcium mobilization was not observed with the Rho kinase inhibitor Y-27632, confirming the notion that Gα13 has the capacity to signal through a Rho kinase-independent process. Although the mechanism for this Gα13-mediated calcium signaling is unknown, it has been suggested to possibly proceed through two separate pathways, i.e. a modulation of calcium channels (36) or through activation of PLC (14). Clearly, additional studies will be required to establish the underlying mechanism(s) involved in this novel signaling process.

Our studies also investigated the role of Gα13 SRI in the PAR1-mediated release reaction. It was found that the secretion of dense granules was considerably more sensitive to inhibition of Gα13 signaling than that observed for either shape change or aggregation. Thus, a concentration of Myr-G13SRI-pep which produced 40% inhibition of shape change and aggregation, resulted in almost complete inhibition of dense granule secretion. Because the platelet release reaction is a complex process involving both inside-out and outside-in signaling, interpretation of this finding is somewhat difficult. Nevertheless, the present results have linked both RhoA activation and calcium mobilization to the Gα13 SRI signaling pathway. Furthermore, it is well established that platelet secretion is in part modulated by intraplatelet calcium levels (49), and that RhoA activation is associated with cytoskeletal rearrangement and secretion (50, 51). Therefore, on this basis, it might be expected that interruption of both signaling pathways may seriously interfere with the release of platelet contents. This finding further suggests the potential importance of the Gα13 signaling pathway in the process of platelet secretion, and perhaps provides a possible explanation of why GPCRs that cannot signal through Gα13, e.g. ADP or epinephrine, also do not cause substantial dense granule release (52, 53). The underlying mechanisms associated with Gα13-mediated secretion will be the basis of future investigations.

The next series of experiments examined the role of Gα13 signaling in thrombin-mediated human platelet aggregation. It was found that Myr-G13SRI-pep produced substantial inhibition of aggregation induced by 0.1 unit/ml thrombin. On the other hand, this inhibitory effect was not observed at higher thrombin doses, i.e. 0.2 units/ml. Because thrombin produces its effects through multiple signaling pathways, interpretation of these data is again somewhat complicated. Thus, in addition to signaling through PAR1 and PAR4 receptors, thrombin can also interact with glycoprotein Ib, leading to activation of α1bβ3 and platelet aggregation. Consequently, at least three separate signaling pathways are involved in the overall thrombin response.

Regarding the GPCR pathways, two principal G protein families participate in the signaling process, i.e. G13 and Gq. Previous results have indicated that PAR1 is coupled to both of these G proteins (12, 54). Furthermore, it has been suggested that PAR4 receptors also couple to G12/13 and Gq (19). Consequently, the composite thrombin response would represent contributions from both G protein pathways. On the other hand, the capacity of these GPCRs to couple to the same G proteins does not necessarily imply that G13 and Gq are equally distributed among PAR1 and PAR4 or that each GPCR signals equally through each G protein (55). Consequently, PAR1 may preferentially signal through G13 and PAR4 may preferentially signal through Gq or vice versa. Our results demonstrate that interruption of G13 signaling has dramatic effects on PAR1-mediated aggregation, suggesting that PAR1 signaling heavily depends on this G protein pathway and that its signaling through Gq is not sufficient to support platelet aggregation. Furthermore, our results provide evidence that PAR4 has a significantly different signaling profile, because aggregation induced by the PAR4 peptide (100 μM AYPGKF) is not blocked by Myr-G13SRI-pep even at the highest peptide concentration tested (i.e. 40 ± 5% aggregation for 500 μM Myr-G13SRI-pep and 45 ± 5% aggregation for 500 μM...
Myr-G13SRI_{Random-pep} \, N = 4). These latter findings indicate that PAR4 induces human platelet aggregation through a largely G13-independent mechanism, which is presumably through Gq. Taken together, these results therefore suggest two important components of thrombin-mediated human platelet aggregation. First, low doses of thrombin selectively activate PAR1 and such activation primarily proceeds through G13 signaling. Second, higher thrombin concentrations cause activation of PAR4, which mediates signaling primarily through the Gq pathway. Thus, removal of the G13 subunit domain plays a role in G13 signaling in human platelets, HEK cells, and human live microvascular endothelial cells, in part through its interaction with p115RhoGEF. In addition, the results provide the first documentation that G13 signaling is required for PAR1-mediated human platelet shape change, aggregation, and secretion. A similar dependence on G13 signaling was also observed for platelet activation by low doses of thrombin. Finally, our results demonstrate that G13 can signal through Rho kinase-independent mechanisms involving the mobilization of intraplatelet calcium. Based on the results obtained with Myr-G13SRI_{pep}, it is believed that the development of additional SRI peptides targeted against other Gq subunits will be of significant value to the study of individual G protein signaling pathways.

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