**Ga13 Switch Region 2 Binds to the Talin Head Domain and Activates αIIbβ3 Integrin in Human Platelets**

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Background: Switch Regions of G protein Ga subunits activate downstream cell signaling events.

Results: Ga13 Switch Region 2 forms a calcium-dependent bi-molecular complex with the head domain of talin.

Conclusion: Binding of the Ga13 to the talin head domain promotes αIIbβ3 integrin activation.

Significance: These results provide a new paradigm for inside-out signaling and αIIbβ3 integrin activation in platelets.

Even though GPCR signaling in human platelets is directly involved in hemostasis and thrombus formation, the sequence of events by which G protein activation leads to αIIbβ3 integrin activation (inside-out signaling) is not clearly defined. We previously demonstrated that a conformationally sensitive domain of one G protein, i.e. Ga13 switch region 1 (Ga13SR1), can directly participate in the platelet inside-out signaling process. Interestingly however, the dependence on Ga13SR1 signaling was limited to PAR1 receptors, and did not involve signaling through other important platelet GPCRs. Based on the limited scope of this involvement, and the known importance of Ga13 in hemostasis and thrombosis, the present study examined whether signaling through another switch region of Ga13, i.e. Ga13 switch region 2 (Ga13SR2) may represent a more global mechanism of platelet activation. Using multiple experimental approaches, our results demonstrate that Ga13SR2 forms a bi-molecular complex with the head domain of talin and thereby promotes β3 integrin activation. Moreover, additional studies provided evidence that Ga13SR2 is not constitutively associated with talin in unactivated platelets, but becomes bound to talin in response to elevated intraplatelet calcium levels. Collectively, these findings provide evidence for a novel paradigm of inside-out signaling in platelets, whereby β3 integrin activation involves the direct binding of the talin head domain to the switch region 2 sequence of the Ga13 subunit.

Cells possess multiple G protein signaling pathways that contribute to the different cellular responses involved in development, function, survival, and disease (1, 2). Consequently, G protein signaling remains an area of intense investigation. It is well established that classic heterotrimeric G protein signaling first requires ligand binding to a G-protein-coupled receptor. The G-protein subunits (Ga, GB, and Gγ) then become activated and interact with downstream effectors to initiate signal transduction events (3, 4). In this regard, the conformationally sensitive switch regions 1 and 2 of the Ga subunit are known to be directly involved in G protein activation and participate in its downstream signaling mechanisms (5–7). Ga13 is one such G protein that is ubiquitously expressed and abundant in all cell types. It is known to be essential for numerous in vivo processes including embryogenesis, angiogenesis, chemokinesis, hemostasis, and thrombosis (2–4). In this connection, we previously demonstrated that human platelet shape change, aggregation, and secretion can be dependent on Ga13 switch region 1 (Ga13SR1)3 signaling (8). However, these studies also provided evidence that the critical importance of this Ga13SR1 signaling pathway is limited to PAR1-mediated platelet activation. Based on this consideration, the present study examined whether a separate Ga13 switch region signaling mechanism, i.e. Ga13SR2 may explain the global importance of Ga13 for in vivo platelet function. Using peptide affinity chromatography of native platelet proteins and immunoaffinity purification of native platelet Ga13-protein interactions, our results demonstrate that the amino acid sequence of Ga13SR2 (but not Ga13SR1) directly binds to the talin-αIIbβ3 integrin-kindlin-3 complex in human platelets. Furthermore, dissociation of this complex revealed that the binding partner for Ga13SR2 is the head domain (also designated as FERM domain) of talin (and not αIIb, β3 integrin or kindlin-3). Importantly, this Ga13SR2-talin binding interaction was promoted by increased intraplatelet calcium levels and prevented by calcium chelation. The ability of talin to form a specific complex with Ga13SR2 was further confirmed by bi-molecular binding measurements using recombinant talin head domain, Ga13SR2 peptides and GST-Ga13SR2 fusion proteins. Lastly, studies measuring fibronectin adhesion of NIH3T3 fibroblasts suggest that the binding interaction between talin and Ga13SR2 is not limited to platelet signaling, but may represent a more universal mechanism of integrin activation.

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* The abbreviations used are: SR, switch region; talinH, talin head domain; PRP, platelet-rich plasma; ASA, aspirin.
**Experimental Procedures**

**Reagents**—Human platelet concentrates (PRP) were purchased from Life Source Blood Services (Glenview, IL). The G13SR2 pep (Myr-VGGQASERKRFWEFCFDS), the G13SR2 mutant pep (Myr-VGGQASERKRWFEFCFDS), the G13SR2 mutant pep (Myr-VGGQASERKDWEFCFDS), the G13SR2 random pep (Myr-GEDEEWEVFKGCQRSS), the G13SR1 pep (Myr-LLARRPTAGI_hey), the G13SR1 random pep (Myr-LIRPTHRHTRLEG), the TRAP1-peptide (SFLLR NPNDKYPEF), the TRAP4-peptide (AYPGKF) and all biotinylated peptide derivatives were synthesized and HPLC purified (>95% pure) by the Research Resource Center, University of Illinois, Chicago. Reagents were from the following sources: ADP and dimethyl-BAPTA-AM (Invitrogen); U46619 (Cayman Chemical); polyclonal rabbit anti-kindlin-3 and the monoclonal anti-αIIb, anti-β3, whole talin antibodies, and fibronectin (Abcam); HRP-conjugated goat anti-rabbit antibody (Cell Signaling); BCA protein assay kit and nitrocellulose membranes (Bio-Rad), Pierce Supersignal kit, TMB and ECL chemiluminescent substrates (Pierce Biochemicals); Streptavidin-HRP (Life Technologies); nitrocellulose blotting membranes, pGEX6p2, and glutathione-Sepharose 4B resin (GE Life Sciences); IPTG and nickel metal affinity chromatography (GoldBio); Src ELISA activation assay kit (Millipore); RhoA G-LISA™ activation assay kit and cell lysis buffer (Cytoskeleton); SulfoLink immobilization kit for peptides and the FITC-PAC1 antibody (Thermo Fisher Scientific); PAC1 monoclonal antibody (Biolegend); protein A-Sepharose beads (Sigma-Aldrich); trypsin EDTA (Corning); Rap1 antibody (Bethyl Laboratories); Go13, His-probe, and GST antibody (Santa Cruz Biotechnology); GFP-C1 plasmid (Clontech Laboratories, Inc); Immulon 2 Removawells (Dynatech Laboratories, Inc); The TA205 antibody was a generous gift from Dr. Stephen Lam (University of Illinois). GFP tagged Full length Talin plasmid was a generous gift from Dr. Jun Qin (Cleveland Clinic, Lerner Research Institute). All reagents used were of analytical grade.

**Human Platelet Functional Studies**—The platelet count in the freshly drawn PRP was adjusted to 3 × 10^8 platelets/ml with calcium-free Tyrode’s buffer (pH 7.4). Platelets were pre-incubated with peptides or vehicle for 1 min prior to incubation with U46619, TRAP1, TRAP4, ADP, or A23187. Aggregation was measured using the turbidimetric method (9), with a model 400 Chrono-Log aggregometer.

**Solubilized Platelet Membrane Preparation**—Solubilized platelet membranes were prepared as previously described (10). Briefly, platelets were sonicated and the membranes were sedimented by ultracentrifugation (100,000 × g) for 45 min at 4 °C. The membrane pellet was then solubilized in cold buffer (25 mM Tris-HCl, 5 mM MgCl₂, pH 7.4, plus 10 mM CHAPS and 5 mM DTT).

**Ca²⁺ Mobilization Assay**—Human PRP was diluted to 2.5 × 10⁶ platelets/ml in Tyrode’s buffer containing 0.1% BSA. Cells were loaded for 1 h at 37 °C with FLIPR calcium-sensitive dye, according to the manufacturer’s protocol. The samples were measured using a FlexStation plate reader (Molecular Devices). Cells were excited at 485 nm, and Ca²⁺ fluorescence was detected at an emission wavelength of 525 nm (11).

**BAPTA Loading of Platelets**—Platelets in PRP were loaded with diethyl-BAPTA-AM (15 μM) for 30 min at 37 °C.

**RhoA G-LISA Activation**—RhoA activation was measured with a G-LISA™ assay. The platelets were treated with agonists alone or were pre-incubated with 250 μM peptide prior to stimulation with the agonists. After 5 min, platelet lysates were prepared, and the bound active Rho-family protein was detected at 490 nm.

**Src ELISA Activation**—Src activation was measured with a Src ELISA activation assay. The platelets were treated with agonists alone or were pre-incubated with 250 μM of peptide prior to stimulation with the agonists. After 15 min, platelet lysates were prepared, and the bound phosphorylated Src was detected at 450 nm.

**SulfoLink Peptide Affinity Columns**—All the peptides used for affinity chromatography were synthesized with a N-terminal cystine and coupled to the SulfoLink agarose beads according to the manufacturer’s protocol.

**Antibody Affinity Columns**—Antibody against Go13 was raised in rabbits immunized with N-terminal peptide (40–48 amino acid sequence) of Go13 (12). This antibody was used to generate an immunoaffinity protein-A Sepharose column to purify Go13 and its associated proteins from solubilized human platelets. The specific column-bound proteins were eluted using a 2.5 pH glycine buffer into neutralization buffer (pH 8.0).

**Western Blotting**—Solubilized platelet membranes or eluates from the peptide affinity columns were normalized for protein concentration using the BCA protein assay. Samples containing 25–50 μg of protein and molecular weight markers were resolved on 10% SDS/polyacrylamide gels. Primary antibodies (13–15) were used at the following dilutions: anti-talin-head domain (TalinH, aa1–25130) (1:1,000), anti-αIIb-β3 1b integrin cytoplasmic domain (aa715–761) were cloned into pGEX6p2. Full length Talin plasmid was a generous gift from Dr. Jun Qin (Cleveland Clinic, Lerner Research Institute). All reagents used were of analytical grade.

**Flow Cytometry Analysis of Platelets using FITC-labeled PAC1 Antibody**—The platelet count in human PRP was adjusted to 1 × 10⁶ platelets/ml using Tyrode’s buffer (pH 7.4). The platelets were stimulated with 0.5 μM U46619 or 25 μM TRAP1 for 5 min and then labeled for 15 min at room temperature with FITC-conjugated PAC1 antibody against activated αIIbβ3. The samples were then analyzed by flow cytometry in a Beckman Coulter CyAn II Flow Cytometer.
**Gα₁₃ Switch Region 2 Activates αlβ3 in Human Platelets**

CGGGGATCCGTTGCGTAAGGAAGTCTTAggCCTGACGAT- 
CCCGACTGG-3'; and G13SR2random. Rev 5'-CCGGTGCGACTC- 
ACTCGGGGTGCGAACCACCTGGCATGCTAAAGA- 
3'. RALGDS plasmid was a generous gift from Cheryl 
Arrowsmith (Addgene plasmid # 25331). Each plasmid 
was transformed into BL21-DE3 strain Escherichia coli and induced 
with 0.2 mM IPTG at an optimal density of 0.6–1.0 for 3 h at 
37 °C. The pET15b constructs were purified using nickel metal 
affinity chromatography while pGEX6p2 constructs were 
purified using Glutathione Sepharose 4B resin according to 
manufacturer's specifications. Eluted proteins were dialyzed 
overnight in a PBS (pH 7.4) and 5% glycerol solution.

**Dot Blot Assay**—Recombinant proteins were pipetted 
directly onto nitrocellulose blotting membranes, rinsed briefly 
in PBS (pH 7.4) and 0.1% Tween 20 (PBST), and incubated with 
5% Milk-PBST for 1 h at room temperature. The membrane 
was rinsed briefly and incubated with 5% BSA-PBST with 0.5 
μM biotinylated peptide for 1 h at room temperature. Subse- 
sequently, each blot was incubated with 5% BSA-PBST with 
streptavidin-HRP for 30 min at room temperature. Each blot 
was developed using ECL chemiluminescent substrates.

**GST Pull-down Assay**—Recombinant GST-G13SR2 and GST- 
G13SR2random proteins (10 μg each) were incubated with gluta- 
thonine-Sepharose 4B resin in Binding Buffer (50 mM Tris-HCl, 
PH 8, 150 mM NaCl, 2 mM EDTA, 1 mM MgCl₂, 0.1% Nonident 
P-40, 5% BSA) for 1 h at room temperature. Beads were washed 
in the binding buffer twice and incubated with 40 μg of His- 
TalinH for 1 h at room temperature, followed by two washes 
using the wash buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 
mM EDTA, 1 mM MgCl₂, 0.1% Nonident P-40). Samples were 
eluted off beads by boiling in 3X SDS sample buffer. Samples 
were resolved using 10% SDS-PAGE gels, transferred to nitro- 
cellulose membranes, and probed with an anti-His 
head domain antibody.

**Construction of Mouse Gα₁₃ Plasmids**—A 2.8 kb cDNA 
encoding wild-type mouse Gα₁₃ (GeneBank™ ID 057665) sub- 
cloned into the pCMV-SPORT6 vector (clone ID 4918717; 
Open Biosystems). Three new plasmids were then created 
based on this vector by selectively mutating either the Gα₁₃SR2 
Arg227 to Ala or the Gα₁₃SR2Arg232 to Ala using the Stratagene 
II site-directed mutagenesis kit (Agilent Technologies) follow- 
ing the manufacturer's instructions. The primers used for these 
point mutation reactions (R227A, 5'-GGGGTAGTGATGCTGGA- 
CCAGGGCACTGACAGGAAACCGCTG-3' and R232A, 5'- 
GCCAGGGATGTTGACGAAAGTAGTCTATTGA- 
3') were made by Integrated DNA Technologies. All 
plasmids used in experiments were amplified from a5-compete 
tent E. coli cultures (NEB) and then purified using endotoxin- 
free maxiprep kits (Qiagen) and sequenced by the DNA 
Services Facility at the UIC Research Resources Center.

**Cell Culture and Plasmid Transfection**—Mouse embryonic 
fibroblasts (NIH3T3) were purchased from ATCC and grown in 
Dulbecco's modified Eagle's medium (DMEM) supple- 
mented with 10% calf bovine serum (CBS), 100 units/ml of penicillin and 0.1 mg/ml streptomycin, under 10% CO₂ at 37 °C. 
When the cells reached 30–40% confluence, the plasmid tran- 
sient transfection was carried out using Fugene HD (Promega) 
according to manufacturer's instructions. Forty-eight hours 
after the transfection, the cells were either trypsinized with 
0.25% Trypsin EDTA for the cell adhesion assay or harvested for 
Western blot using cell lysis buffer.

**Cell Adhesion Assay**—Non-treated, Corning® 96 Well Black 
Flat Bottom Polystyrene Microplates were coated with 200 μl of 
mouse fibronectin (10 μg/ml), at 37 °C for 1 h. The plates were 
rinsed with 200 μl of PBS twice, and the nonspecific binding 
sites were blocked with 200 μl heat-treated 1% BSA/PBS solution 
37 °C for 1 h. Cultured NIH3T3 cells were harvested with 
0.25% trypsin EDTA, and the cell suspension was centrifuged 
(80 x g) for 2 min. The pelleted cells were resuspended in 
colorless DMEM (Mediatech, Inc.) at a concentration of 5 x 10⁶ 
cells/ml. 5 μl/ml of calcein AM (Life Technologies) was then 
added, and the cell suspension was incubated at 37 °C for 30 
min. The cells were then separated from the suspension 
medium by centrifugation (80 x g) for 2 min and resuspended 
(5 x 10⁶ cells/ml) in adhesion buffer (Hank's balanced salt solu- 
tion plus 1% BSA). Following resuspension, 100 μl of the cells 
were added to each well, and the plates were incubated for 2 h at 
37 °C. Finally, the wells were washed 4X with PBS buffer read 
for fluorescence at 517 nm using a fluorescein filter.

**Quantification of Western Blots/Dot Blots and Statistical 
Analysis**—NIH software, ImageJ, was used for densitometric 
analysis of blots. Unless otherwise specified, significance was 
determined between samples using unpaired Student’s t test 
(p < 0.05). All results are representative of data obtained using 
least three separate experiments/and or 3 separate units of 
human PRP.

**Rap1 Activity Assay**—Rap1 activity was determined as previously 
described (16). Human PRP was adjusted to 2 x 10⁶ plate- 
lets/ml in Tyrode’s buffer. Platelets were pre-treated with either 
150 μM Myr-G13SR2 pep, 150 μM Myr-G13SR2Random pep, 
DMSO, or 10 ng/ml PGI2 for 2 min prior to stimulation with 
1.0 μM A23187 for 5 min on ice with gentle agitation. Plate- 
lets were immediately lysed using 2X RIPA buffer with Complete 
Protease Inhibitor Mixture (Roche) for 15 min with gentle 
agitation. Clarified lysate was obtained through centrifugation 
at 14,000 x g at 4 °C for 10 min. The supernatant was incubated 
with 5 μg of RalGDS bound to nickel bead resin (GoldBio) for 
90 min at 4 °C with gentle agitation. Resin was washed with 
1X RIPA buffer and eluted by boiling in SDS loading buffer. Bound 
Rap1 was detected via Western blotting using anti-Rap1 
antibody.

**TalinH ELISA**—Recombinant 6x-His tagged Talin head 
domain (talinH) was added to Immulon2 Removawells (Dynatech 
Laboratories, Inc.) in 50 mM sodium carbonate/bicarbonate 
buffer (pH 9.4) and incubated overnight at 4 °C to generate a 
standard curve. Samples were blocked with 5% BSA-PBST for 
1 h at room temperature, washed thoroughly, and incubated 
with an anti-His antibody (Santa Cruz Biotechnology). TalinH 
concentration was determined using TMB substrate at 450 nm. 
Subsequently, GST-G13SR2, GST- G13SR2Random pep and GST 
were added to Immulon 2 wells at concentrations ranging from 
1.0 μg/ml to 1.0 ng/ml and probed with an anti-GST antibody 
(Santa Cruz Biotechnology). Equal loading concentrations were 
normalized by absorbance values. To determine talinH bind- 
ging, GST fusion proteins were added to Immulon2 wells in 
equal concentrations overnight at 4 °C. Each well was blocked
in 5% BSA-PBST, incubated with talinH, and washed thoroughly. TalinH binding was measured by probing with an anti-His antibody and developed using TMB substrate at 450 nm. Each value was converted to picogram of talinH using the linear range of the standard curve. Each analysis was performed in triplicate and statistical significance was determined using unpaired Student’s t test (p < 0.05).

**PAC1 Integrin Activation Assay**—CHO cells stably expressing αIIbβ3 integrins (CHO5 cells) were a generous gift from Dr. Mark Ginsberg. CHO5 cells were transfected with either Ga13 wild type (WT), or Ga13 R227A or R232A mutants. After 24 h, integrin activation was determined using a PAC1 monoclonal antibody (Biolegend) and an Alexa555 conjugated secondary antibody (Life Technologies). Cells were fixed in 0.5% PFA for 15 min at room temperature and washed thoroughly in PBS. Cells were blocked and permeabilized for 30 min on ice using a PBS buffer containing 4% BSA and 0.1% Saponin. After thorough washing in PBS, Ga13 was probed using an anti-Ga13 antibody (Santa Cruz) and an Alexa 405 conjugated secondary antibody (Life Technologies). Cells were analyzed using a BD LSRii flow cytometer and PAC1 activation was measured for all cells and Ga13-positive cells. To evaluate the effect of Talin and Ga13 together, CHO5 cells were co-transfected with Ga13 WT, R227A, or R232A and either GFP (Control) or GFP Talin (Experimental). Integrin activation was determined by gating for Ga13-, GFP-, and PAC1-positive cells. The overall change in percent integrin activation was determined as follows: ∆Activation = (% Experimental/% Control). Each analysis was performed in triplicate and statistical significance was determined using unpaired Student’s t test (p < 0.05).

**Results**

**Agonist Activation of αIIbβ3 Is Dependent on Ga13 Switch Region 2 Signaling**—To investigate Ga13-SR2 signaling in human platelets, a myristoylated peptide comprising the complete amino acid sequence of Ga13-SR2 was synthesized. This peptide was employed as both a competitive inhibitor of Ga13-SR2 signaling in intact platelets and as an immobilized ligand to enable the purification of Ga13-SR2-associated proteins. In the intact platelet studies, it was necessary to demonstrate that the myristoylated Ga13-SR2 peptide gained access to the platelet cytosolic compartment. To this end, human PRP was treated with myristoylated Ga13-SR2 pep non-myristoylated Ga13-SR2 pep (vehicle (DMSO)). After incubation, the PRP samples were centrifuged and the supernatant was removed to obtain the unbound fraction as shown in Fig. 1a (left panel). The pellet was then lysed and centrifuged to separate the membrane and cytosolic fractions as shown in the middle and right panel of Fig. 1a, respectively. Western blotting data clearly show that the non-myristoylated peptide is primarily located in the unbound fraction, while the myristoylated peptide is primarily taken up into the cytosolic fraction. These results demonstrate that myristoylated Ga13-SR2 pep permeates the platelet plasma membrane and has access to intraplatelet signaling processes. The next series of experiments examined whether this Ga13-SR2 peptide (Ga13-SR2 pep) interfered with human platelet aggregation in PRP. It was found (Fig. 1b) that Ga13-SR2 pep blocked platelet aggregation (trace c) stimulated by all the agonists examined, including U46619, TRAP1, ADP, TRAP4, and A23187. Clearly, this inhibitory profile is quite different from that associated with a peptide representing Ga13-SR1 (Ga13-SR1 pep), which only blocked aggregation mediated by TRAP1 (trace b). Furthermore, a single change in the amino acid sequence of Ga13-SR2 pep at amino acid 227 (from Arg to Ala; Ga13-SR2227mutant pep) significantly reduced its inhibitory effects (trace d), demonstrating the high degree of specificity of Ga13-SR2 pep. To investigate the mechanism by which Ga13-SR2 pep blocks platelet aggregation, we first examined its role in primary platelet aggregation stimulated by ADP (3 μM) in human PRP. Specifically, aspirin (ASA) treatment was used to block the secondary aggregation process that derives from the secretion of platelet dense granules. The results (aggregation upper panel and secretion lower panel) are presented in Fig. 1c. Trace e and bar e illustrate the ADP-mediated aggregation and secretion response in PRP pretreated with 75 μM Ga13-SR2 pep alone. It can be seen that pretreatment of the PRP with 1.0 mM ASA resulted in partial inhibition of the aggregation (upper panel, trace f) and a complete inhibition of dense granule secretion (lower panel, bar f), relative to no ASA treatment (trace e and bar e). Thus, in the presence of 1.0 mM ASA, the residual aggregation (trace f) represents the primary aggregation response. Furthermore, the results illustrate that Ga13-SR2 pep (75 μM) caused almost complete inhibition of the residual, ADP-induced, primary aggregation response (upper panel, trace g), even though it only produced partial inhibition of secretion (lower panel, bar g). Finally, when platelets were treated with both 1.0 mM ASA and 75 μM Ga13-SR2 pep, both aggregation (trace h) and secretion (bar h) were completely blocked. Similar results were obtained using A23187 under the same experimental conditions (data not shown). These data demonstrate that Ga13-SR2 pep Peptide is capable of blocking primary human platelet aggregation.

The next experiments investigated the fundamental differences between Ga13-SR1 and Ga13-SR2 signaling pathways by comparing the abilities of Ga13-SR1 pep and Ga13-SR2 pep to modulate downstream platelet effectors. It was found that Ga13-SR1 pep substantially blocked RhoA, calcium, and Src activation induced by TRAP1 but not by U46619. Surprisingly however, Ga13-SR2 pep did not block these signaling pathways induced by either TRAP1 or U46619 (Fig. 1d). These findings indicate that Ga13-SR1 and Ga13-SR2 have completely different signaling profiles in human platelets, and that unlike Ga13-SR1 signaling, the signaling events mediated by Ga13-SR2 lie further downstream from RhoA, calcium mobilization, or Src.

Based on these findings, experiments were undertaken to investigate whether Ga13-SR2 pep interferes with a separate and well-known mediator of platelet aggregation, i.e. the Rap1/RIAM signaling pathway. To examine this possibility, human PRP was activated with 1.0 μM A23187 to mobilize calcium, and treated with either PGI2 to inhibit Rap1 activation, or with Ga13-SR2 pep or Ga13-SR2 random pep. After treatment, the platelets were lysed and incubated with Rap1GDS to harvest active GTP-bound form of Rap1 that was in turn detected using immunoblotting with a Rap1 antibody (Fig. 1, panel e). The lower panel of blots show total Rap1 in PRP, while the upper panel of blots show the active form of Rap1 upon each treatment (Fig. 1). It is...
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Effects of Gα13SR1 random and Gα13SR2 random on human platelet function. a, myristoylated or non-myristoylated peptide were incubated with platelet PRP, which was then separated into unbound, membrane bound, or cytosolic fractions. An antibody raised against Gα13SR2 sequence was used for immunoblotting.

Effects of Gα13SR1 pep and Gα13SR2 pep on human platelet function. a, myristoylated or non-myristoylated peptide were incubated with platelet PRP, which was then separated into unbound, membrane bound, or cytosolic fractions. An antibody raised against Gα13SR2 sequence was used for immunoblotting.

clear that A23187 increases the amount of active Rap1 as compared with platelets treated with vehicle (DMSO), while PGI_2 treatment abrogates Rap1 activation in agreement with previous findings (16). Finally, when pre-treated with Gα13SR2 pep or Gα13SR2 random pep the activity of Rap1 remained unchanged. Together, these data indicate that Gα13SR2 pep acts downstream of the calcium mobilization and Rap1 pathways.

On this basis, we next examined the possibility that Gα13SR2 modulates a later step in the platelet activation process, i.e., αIIbβ3 integrin activation (17). These experiments employed FITC-PAC1 antibody and flow cytometry analysis to measure agonist-mediated activation of platelet αIIbβ3 (18). It was found that Gα13SR2 pep did indeed inhibit αIIbβ3 activation in response to both TRAP1 (Fig. 1j) and U46619 (Fig. 1g). Thus, while Gα13SR1 signaling induces calcium mobilization, RhoA activation and Src activation specifically through PAR1 (19) receptor stimulation, Gα13SR2 signaling is recruited by multiple platelet receptors and acts at a further downstream target involving αIIbβ3 activation.

Gα13 Switch Region 2 Interacts with the Talin-αIIbβ3-kindlin-3 Complex by Binding to the Talin Head Domain—To identify the mechanism by which Gα13SR2 modulates αIIbβ3 function, we performed experiments to determine whether the amino acid sequence of Gα13SR2 interacts with αIIbβ3 integrin or an αIIbβ3-associated protein such as talin (20). To this end, peptide-affinity chromatography was employed. Briefly, Gα13SR2 pep was coupled to a SulfoLink agarose column to purify Gα13SR2-associated proteins from solubilized human platelets. Western blot analysis of the affinity eluates from the Gα13SR2 pep column demonstrated the purification of intact talin, αIIbβ3, and kindlin-3 (Fig. 2a). The specificity of this affinity purification was demonstrated by the finding that an affinity column using Gα13SR2 random pep did not purify any of these platelet proteins (Fig. 2a). These findings provide evidence that the amino acid sequence comprising Gα13SR2 directly binds to a protein complex (13, 21–26) containing talin, αIIbβ3, and kindlin-3. This notion was further confirmed by measuring the association of endogenous platelet Gα13 with
this protein complex. Specifically, an antibody generated against the N-terminal peptide sequence of G\(\alpha_{13}\) (12) was coupled to an immunoaffinity column (G\(\alpha_{13}\)-Ab) and used to purify G\(\alpha_{13}\)-associated proteins from solubilized platelets. Western blot analysis of the eluates from the G\(\alpha_{13}\)-Ab immunoaffinity column again demonstrated the purification of talin, \(\alpha llb\), and kindlin-3 (Fig. 2b), whereas an immunoaffinity column using the pre-immune IgG did not result in the purification of any of these proteins (Fig. 2b).

The next series of experiments were designed to identify the binding partner of G\(\alpha_{13}\) within the protein complex. Our data demonstrate that G\(\alpha_{13}\) binds to talin, but not to \(\alpha llb\), \(\beta 3\), integrin or kindlin-3. Specifically, when the talin-\(\alpha llb\)-kindlin-3 complex was dissociated by heating the solubilized platelet membrane samples at 100 °C for 45 min prior to affinity chromatography. The resulting eluates from the G\(\alpha_{13}\)SR2pep (or G\(\alpha_{13}\)SR2\(\alpha llb\)pep) columns (panel c) and the G\(\alpha_{13}\)Ab immunoaffinity (or control pre-immune IgG) column (panel d) were immunoblotted for talin, \(\alpha llb\), \(\beta 3\), and kindlin-3. In panel e the solubilized platelet membrane eluates from the G\(\alpha_{13}\)Ab immunoaffinity column or the control pre-immune IgG column were immunoblotted for the talin head domain. In panel f competition binding was performed by incubating solubilized platelet membranes with 250 \(\mu M\) of G\(\alpha_{13}\)SR2pep (or G\(\alpha_{13}\)SR2\(\alpha llb\)pep) as the competing agent prior to G\(\alpha_{13}\)Ab immunoaffinity column chromatography. In panel g the solubilized platelet membrane eluates from an affinity columns using G\(\alpha_{13}\)SR2pep or a mutant peptide with a single amino acid substitution (R227A; G\(\alpha_{13}\)SR2227 mutant pep) were immunoblotted for the talin head domain. In panel h the solubilized platelet membrane eluate from a peptide affinity columns using G\(\alpha_{13}\)SR1pep was immunoblotted for whole talin, \(\alpha llb\), \(\beta 3\), kindlin-3, and p115-RhoGEF. In cases where the gel was probed with more than one antibody, the panel represents a composite of separate blots where each lane was run using its own molecular weight standards. All results are representative of data obtained using at least three separate units of human PRP, and representative data were chosen for each panel.
well as the talin rod domain (21), the absence of the talin rod domain (Fig. 2, a and b) suggests that the head domain of talin serves as the binding region for Ga\textsubscript{\alpha13}. This finding was confirmed by blotting the Ga\textsubscript{\alpha13}-Ab immunoaffinity column eluate with the TA205 antibody that specifically recognizes the talin head domain (13) (Fig. 2e). Evidence that the binding of Ga\textsubscript{\alpha13} to the talin head domain is mediated through the Ga\textsubscript{\alpha13}SR2 sequence was provided by binding competition studies. In these experiments, solubilized platelet membranes were pre-incubated with G\textsubscript{\alpha13}SR2pep before protein purification by the Ga\textsubscript{\alpha13}-Ab immunoaffinity column. The results demonstrated that competition with G\textsubscript{\alpha13}SR2pep inhibited native Ga\textsubscript{\alpha13} binding to the talin head domain (Fig. 2f). Taken together, these findings suggest that Ga\textsubscript{\alpha13} specifically binds to the talin head domain through the G\textsubscript{\alpha13}SR2 sequence, and does not directly associate with either \textalpha\textbetallb, \beta3 integrin, or kindlin-3. Furthermore, the aggregation data shown in Fig. 1a suggest that Arg-227 within the G\textsubscript{\alpha13}SR2 sequence may be important for talin binding. This notion was confirmed by the finding that an affinity column using the G\textsubscript{\alpha13}SR2\textsubscript{227 mutant pep} (R227A) was not effective in purifying the talin head domain (Fig. 2g).

Our previous results demonstrated that G\textsubscript{\alpha13} Switch region 1 (G\textsubscript{\alpha13}SR1) binds to and signals through p115-RhoGEF in human platelets. In order to examine whether G\textsubscript{\alpha13} SR1 also interacts with the \textalpha\textbetallb3-kindlin-3 complex, we again employed peptid-amine affinity chromatography as described above. In this case, the column was prepared using G\textsubscript{\alpha13}SR1pep coupled to SulfoLink agarose beads. However, Western blot analysis of the affinity eluates from the G\textsubscript{\alpha13}SR1pem column demonstrated only the purification of p115-RhoGEF and not the purification of talin, \textalpha\textbetallb, \beta3 integrin, or kindlin-3 (Fig. 2h).

Additional experiments provided independent confirmation of the direct binding interaction between G\textsubscript{\alpha13} SR2 and the talin head domain. To this end, we employed a bi-molecular in vitro binding reaction outside of the platelet milieu. Using a dot blot assay, it was found that recombinant talin head domain directly binds to G\textsubscript{\alpha13}SR2pem but not to the G\textsubscript{\alpha13}SR2\textsubscript{random pep} (Fig. 3, a and b). Moreover, the specificity of G\textsubscript{\alpha13}SR2pem-talin binding complex was demonstrated by using two different G\textsubscript{\alpha13}SR2 mutant peptides, each with a single R to A substitution at positions 227 or 232 (G\textsubscript{\alpha13}SR2\textsubscript{227 mutant pep} and G\textsubscript{\alpha13}SR2\textsubscript{232 mutant pep}). The results demonstrated (Fig. 3, a and b) that each mutant exhibited a significantly reduced binding affinity for talin head domain. To further demonstrate the specificity of this binding interaction between G\textsubscript{\alpha13} SR2 and talin head domain, we utilized an alternate experimental approach. Specifically, recombinant GST-G\textsubscript{\alpha13}SR2 and GST-G\textsubscript{\alpha13}SR2\textsubscript{random} fusion proteins were generated and incubated with recombinant talin head domain to quantify their binary interactions. The results show that while GST-G\textsubscript{\alpha13}SR2 bound to talin, GST-G\textsubscript{\alpha13}SR2\textsubscript{random} did not (Fig. 3c). Taken together, these results add additional support to the notion that G\textsubscript{\alpha13} SR2 can form a bi-molecular complex with the talin head domain.

G\textsubscript{\alpha13} Switch Region 2 Directly Binds to Talin but Not to \beta3 Integrin—Our previous experiments (Fig. 2d) indicated that G\textsubscript{\alpha13} is capable of forming a ternary complex with talin but not with \beta3 integrin. This notion was further investigated by additional dot blot binding assays measuring the ability of biotinylated G\textsubscript{\alpha13}SR2\textsubscript{pep} (or G\textsubscript{\alpha13}SR2\textsubscript{random pep}) to bind with recombinant GST-talin head domain, GST-\beta3 cytoplasmic tail, or GST control. It was found that G\textsubscript{\alpha13}SR2\textsubscript{pep} bound only to GST-talin head domain, but not to the GST-\beta3 cytoplasmic tail (Fig. 3, d and e). Furthermore, the specificity of the binding interaction between G\textsubscript{\alpha13} SR2 and talin head domain was again demonstrated by the finding that biotinylated G\textsubscript{\alpha13}SR1pem bound to neither GST-talin head domain nor GST-\beta3 cytoplasmic tail (Fig. 3, d and e), but did effectively bind to recombinant full-length p115-RhoGEF (Fig. 3f). This preference of G\textsubscript{\alpha13}SR1 for binding to p115-RhoGEF is consistent with our findings in solubilized platelet membranes (Fig. 2h) and our previously published results (8). To demonstrate that the recombinant GST-\beta3 integrin cytoplasmic tail employed in the previous experiment (Fig. 3, d and e) was indeed functional, we tested the binding activity of GST-\beta3 integrin cytoplasmic tail with talin head domain (22). As expected, talin head domain significantly bound GST-\beta3 integrin (Fig. 3g) under the same experimental conditions as employed in Fig. 3, d and e. Finally, an ELISA was used to examine Ga\textsubscript{\alpha13} SR2 interaction with talin. Using a talin H standard curve (Fig. 3h), the picograms of talin bound to GST, G\textsubscript{\alpha13}SR2pem or GST- G\textsubscript{\alpha13}SR2\textsubscript{random pep} is illustrated in Fig. 3i. Collectively, the above findings provide strong evidence that G\textsubscript{\alpha13}SR2 mediates integrin activation by directly binding to the talin head domain and not to the \beta3 integrin cytoplasmic tail.

Intraplatelet Calcium Stimulates the Binding of Ga\textsubscript{\alpha13} Switch Region 2 to Talin—We next examined a possible mechanistic basis for the binding interaction between Ga\textsubscript{\alpha13} SR2 and talin head domain. Since intraplatelet calcium is known to be a common mediator of \beta3 integrin activation, we first tested the effects of the calcium ionophore A23187 on Ga\textsubscript{\alpha13}-talin binding. Specifically, intact human platelets (PRP) were treated with 10 \textmu M A23187, solubilized, and then subjected to Ga\textsubscript{\alpha13}-Ab immunoaffinity chromatography as described above. The results demonstrated (Fig. 4a) that elevation of intraplatelet calcium by A23187 caused a notable increase in Ga\textsubscript{\alpha13}-talin head domain binding. This observation was further confirmed by chelating available calcium with EGTA and BAPTA. Under these conditions, the binding of Ga\textsubscript{\alpha13} to talin was completely abolished, even in the presence of A23187 (Fig. 4a). These results indicate that Ga\textsubscript{\alpha13} is not constitutively associated with talin in truly un-activated platelets, but only becomes bound to talin in the presence of elevated calcium.

G\textsubscript{\alpha13} Switch Region 2 Signaling Is Essential for Adhesion of NIH3T3 Cells—To determine whether Ga\textsubscript{\alpha13} SR2-talin signaling is limited to human platelets or whether it may serve as a more global mechanism of integrin activation, cell adhesion experiments were performed using fibroblasts. Specifically, we expressed both wild-type and mutant forms of the Ga\textsubscript{\alpha13} protein in cultured NIH3T3 cells and measured the effects of these Ga\textsubscript{\alpha13} SR2 mutations on cell adhesion to fibronectin. The rationale for these experiments derives from our previous observations indicating that arginine 227 and arginine 232 contained within Ga\textsubscript{\alpha13} SR2 each play a critical role in Ga\textsubscript{\alpha13} SR2-talin binding and integrin activation. Thus, if Ga\textsubscript{\alpha13} SR2 signaling is in fact essential for the process of cell adhesion, overexpression of the mutant forms of Ga\textsubscript{\alpha13} should interfere with this process, due to the loss of important arginine residues within the Ga\textsubscript{\alpha13} SR2...
sequence. The results from these experiments are consistent with this notion. Specifically, Western blot analysis (Fig. 4b) of transfected NIH3T3 cells demonstrated that the expression levels of the wild-type Gα13 were comparable to the expression levels of both the Gα13 R227A and the Gα13 R232A mutant proteins. However, despite these equal expression levels, the Gα13SR2 mutant (R227A and R232A) cells were significantly less capable of adhering to fibronectin compared with the wild-


**FIGURE 4. Effect of G13, on integrin activation.** Fig. 4 panel a shows that increased intraplatelet calcium levels promote native G13-talin binding. Platelets from untreated human PRP or A23187 (10 μM)-treated PRP were solubilized and subjected to G13-Ab immunoaffinity column chromatography. In separate experiments, the PRP was supplemented with EGTA (3 mM) and the platelets were loaded with BAPTA prior to A23187 (10 μM) stimulation. The platelets were then solubilized and subjected to G13-Ab immunoaffinity column chromatography. G13 blotting, shown in the left two lanes, was used as a loading control. All results are representative of data obtained using at least 3 separate units of human PRP, and representative data were chosen. In panel b cultured mouse embryonic fibroblasts (NIH3T3) were transfected with plasmids containing G13, wild-type, G13, R227A, or G13, R232A. Forty-eight hours after transfection, the cells were harvested for Western blot analysis of G13, or allowed to adhere to fibronectin-coated plates. The Western blots illustrated in the top of the figure illustrate comparable levels of G13 expression in each case. In panel c a G13 plasmid or vector alone was transfected into CHO cells stably expressing platelet αIIbβ3 integrins (CHOa5). Percent integrin activation was determined using a monoclonal PAC1 antibody. In panel d the G13, wild-type, G13, R227A, or G13, R232A plasmids were transfected into CHOa5 cells, gated for G13 expression and the percent of cells with active integrins was quantified. In panel e each G13 construct was co-transfected with either GFP or GFP tagged full-length talin and the change in integrin activation between G13, co-expression with GFP and GFP-talin. Significance (*) (p < 0.05) was determined by ANOVA using GraphPad PRISM 5.04 statistical software (San Diego, CA). All results are representative of at least three separate experiments (n = 3).

**Discussion**

In the present study, the underlying basis for platelet inside-out signaling has been further investigated by examining a direct mechanistic link between G13 signaling and αIIbβ3 integrin activation. The results demonstrate evidence for such a link involving switch region 2 of the G13 subunit. Our past (8) and present findings suggest that G13 actually plays two important and distinct roles in the platelet activation process, depending on whether signaling occurs through its Switch Region 1 or through its Switch Region 2 (see model in Fig. 5). In this regard, we found that signaling through G13,SR1 is a classical GPCR-linked event that derives from agonist interaction with a G13-coupled receptor. The activated G13,SR1 then causes stimulation of multiple downstream effectors, including RhoA, calcium, and Src, among others. The specificity of this signaling pathway is consistent with the notion that inhibition of G13,SR1 signaling has very selective and limited effects on platelet function. Thus, G13,SR1 inhibitors only block signaling by GPCRs that are primarily coupled to G13, i.e. PAR1 (Fig. 1b, trace b) (8), and do not block platelet aggregation in response to other platelet agonists, e.g. ADP, TRAP4, U46619, A23187, etc., because these agonists do not heavily rely upon
As a basis for \( \text{G}_{13} \) signal transduction (8). Our present and previous demonstration that \( \text{G}_{13\text{SR}1} \) signaling is specific for PAR1-mediated activation is consistent with recent work (27, 28) describing a binding interaction between \( \text{G}_{13} \) and PAR1 activation and subsequent downstream signaling events, e.g. calcium mobilization, RhoA and Src activation, etc. In contrast, signaling through \( \text{G}_{13\text{SR}2} \) is not directly GPCR-dependent, but rather calcium-dependent. In this case, elevation of intraplatelet calcium promotes the recruitment of talin by the CIaDAG-GEF/Rap1/RIAM pathway, binding of \( \text{G}_{13} \) to whole talin, cleavage of the talin rod domain, binding of the \( \text{G}_{13} \)-talin head complex to the cytoplasmic tail of \( \beta 3 \) integrin, and dissociation of \( \text{G}_{13} \) from the talin head complex. Because of these significant differences in signaling mechanisms, selective inhibition of \( \text{G}_{13\text{SR}1} \) and \( \text{G}_{13\text{SR}2} \) signaling produces markedly different effects on human platelet function. Thus, inhibition of \( \text{G}_{13\text{SR}1} \) signaling only blocks PAR1-mediated platelet aggregation, whereas inhibition of \( \text{G}_{13\text{SR}2} \) signaling blocks aggregation in response to all platelet agonists.

**FIGURE 5.** A model for two separate \( \text{G}_{13\text{SR}1} \) Switch Region signaling pathways. \( \text{G}_{13\text{SR}1} \) is a classical receptor-coupled signal transduction pathway where agonist interaction with a surface membrane GPCR leads to \( \text{G}_{13\text{SR}1} \) activation and subsequent downstream signaling events, e.g. calcium mobilization, RhoA and Src activation, etc. In contrast, signaling through \( \text{G}_{13\text{SR}2} \) is not directly GPCR-dependent, but rather calcium-dependent. In this case, elevation of intraplatelet calcium promotes the recruitment of talin by the CIaDAG-GEF/Rap1/RIAM pathway, binding of \( \text{G}_{13} \) to whole talin, cleavage of the talin rod domain, binding of the \( \text{G}_{13} \)-talin head complex to the cytoplasmic tail of \( \beta 3 \) integrin, and dissociation of \( \text{G}_{13} \) from the talin head complex. Because of these significant differences in signaling mechanisms, selective inhibition of \( \text{G}_{13\text{SR}1} \) and \( \text{G}_{13\text{SR}2} \) signaling produces markedly different effects on human platelet function. Thus, inhibition of \( \text{G}_{13\text{SR}1} \) signaling only blocks PAR1-mediated platelet aggregation, whereas inhibition of \( \text{G}_{13\text{SR}2} \) signaling blocks aggregation in response to all platelet agonists.

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