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G protein subunit Gα_{13} binds to integrin $\alpha_{IIb}\beta_3$ and mediates integrin "outside-in" signaling

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Abstract

Integrins mediate cell adhesion to the extracellular matrix and transmit signals within the cell that stimulate cell spreading, retraction, migration, and proliferation. The mechanism of integrin outside-in signaling has been unclear. We found that the heterotrimeric guanine nucleotide-binding protein (G protein), Gα_{13}, directly bound to the integrin β_3 cytoplasmic domain, and that Gα_{13}-integrin interaction was promoted by ligand binding to the integrin $\alpha_{IIb}\beta_3$ and by guanosine triphosphate (GTP)-loading of Gα_{13}. Interference of Gα_{13} expression or a myristoylated fragment of Gα_{13} that inhibited interaction of $\alpha_{IIb}\beta_3$ with Gα_{13} diminished activation of protein kinase c-Src and stimulated the small GTPase RhoA, consequently inhibiting cell spreading and accelerating cell retraction. We conclude that integrins are non-canonical Gα_{13}-coupled receptors that provide a mechanism for dynamic regulation of RhoA.

Integrins mediate cell adhesion and transmit signals within the cell that lead to cell spreading, retraction, migration, and proliferation (1). Thus, integrins have pivotal roles in biological processes such as development, immunity, cancer, wound healing, hemostasis and thrombosis. The platelet integrin, $\alpha_{IIb}\beta_3$, typically displays bidirectional signaling function (2,3). Signals from within the cell activate binding of $\alpha_{IIb}\beta_3$ to extracellular ligands, which in turn triggers signaling within the cell initiated by the occupied receptor (so-called "outside-in" signaling). A major early consequence of integrin "outside-in" signaling is cell spreading, which requires activation of the protein kinase c-Src and c-Src-mediated inhibition of the small guanosine triphosphatase (GTPase) RhoA (4-7). Subsequent cleavage of the c-Src binding site in β_3 by calpain allows activation of RhoA, which stimulates cell retraction (7,8). The molecular mechanism coupling ligand-bound $\alpha_{IIb}\beta_3$ to these signaling events has been unclear.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) consist of Gα, Gβ and Gγ subunits (9). G proteins bind to the intracellular side of G-protein coupled receptors (GPCR) and transmit signals that are important in many intracellular events (9-11). Gα_{13}, when activated by GPCRs, interacts with Rho guanine-nucleotide exchange factors (RhoGEF) and thus activates RhoA (11-14), facilitating contractility and rounding of discoid platelets (shape change). To determine whether Gα_{13} functions in signaling from ligand-occupied integrin, we investigated whether inhibition of Gα_{13} expression with small interfering RNA (siRNA) affected $\alpha_{IIb}\beta_3$-dependent spreading of platelets on fibrinogen, which is an integrin ligand. We isolated mouse bone marrow stem cells and transfected them with lentivirus encoding Gα_{13}

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siRNA. The transfected stem cells were transplanted into irradiated C57/BL6 mice (15). Four to six weeks after transplantation, nearly all platelets isolated from recipient mice were derived from transplanted stem cells as indicated by the enhanced green fluorescent protein (EGFP) encoded in lentivirus vector (Fig. S1,Fig. 1A). Platelets from $G\alpha_{13}$ siRNA-transfected stem cell recipient mice showed >80% decrease in $G\alpha_{13}$ expression (Fig. 1B). When platelets were allowed to adhere to immobilized fibrinogen [$\alpha_{IIb}\beta_3$ binding to immobilized fibrinogen does not require prior "inside-out" signaling activation (16)], platelets depleted of $G\alpha_{13}$ spread poorly as compared with control platelets (Fig. 1A,Fig. S2). The inhibitory effect of $G\alpha_{13}$ deficiency is unlikely to be caused by its effect on GPCR-stimulated $G\alpha_{13}$ signaling because (i) washed resting platelets were used and no GPCR agonists were added, and (ii) prior treatment with 1 mM aspirin [which abolishes thromboxane A_2 (TXA_2) generation (17)] did not affect platelet spreading on fibrinogen (Fig. S2), making it unlikely the endogenous TXA_2-mediated stimulation of $G\alpha_{13}$. Furthermore, $G\alpha_{13}$ siRNA inhibited spreading of Chinese hamster ovary (CHO) cells expressing human $\alpha_{IIb}\beta_3$ (123 cells) (18), which was rescued by an siRNA-resistant $G\alpha_{13}$ (Fig. S3). Thus, $G\alpha_{13}$ appears to be important in integrin "outside-in" signaling leading to cell spreading.

To determine whether $G\alpha_{13}$ serves as an early signaling mechanism that mediates integrin-induced activation of c-Src, we measured phosphorylation of c-Src at Tyr^{416} (which indicates activation of c-Src) in control and fibrinogen-bound cells. Depletion of $G\alpha_{13}$ in mouse platelets or 123 cells abolished phosphorylation of c-Src Tyr^{416} (Fig. 1C, Fig. S3), indicating that $G\alpha_{13}$ may link integrin $\alpha_{IIb}\beta_3$ and c-Src activation. Because c-Src inhibits RhoA (7,19), we also tested the role of $G\alpha_{13}$ in regulating activation of RhoA. RhoA activity was suppressed to baseline 15 minutes after platelet adhesion, and became activated at 30 minutes (Fig. 1C), which is consistent with transient inhibition of RhoA by c-Src (7). The integrin-dependent delayed activation of RhoA was not inhibited by depletion of $G\alpha_{13}$, indicating its independence of the GPCR-$G\alpha_{13}$-RhoGEF pathway (Fig. 1C). In contrast, depletion of $G\alpha_{13}$ accelerated RhoA activation (Fig. 1C). Thus, $G\alpha_{13}$ appears to mediate inhibition of RhoA. The inhibitory effect of $G\alpha_{13}$ depletion on platelet spreading was reversed by Rho-kinase inhibitor Y27632 (Fig. 1A), suggesting that $G\alpha_{13}$-mediated inhibition of RhoA is important in stimulating platelet spreading. These data are consistent with $G\alpha_{13}$ mediating integrin "outside-in signaling" inducing c-Src activation, RhoA inhibition, and cell spreading.

The integrin $\alpha_{IIb}\beta_3$ was co-immunoprecipitated by anti-$G\alpha_{13}$ antibody, but not control IgG, from platelet lysates (Fig. 2A). Conversely, an antibody to β_3 immunoprecipitated $G\alpha_{13}$ with β_3 (Fig. 2B). Co-immunoprecipitation of β_3 with $G\alpha_{13}$ was enhanced by GTP-γS or AlF_4^- (Fig. 2A, Fig. S4). Thus, β_3 is present in a complex with $G\alpha_{13}$, preferably the active GTP-bound $G\alpha_{13}$. To determine whether $G\alpha_{13}$ directly binds to the integrin cytoplasmic domain, we incubated purified recombinant $G\alpha_{13}$ (20) with agarose beads conjugated with glutathione S-transferase (GST), or a GST-β_3 cytoplasmic domain fusion protein (GST-β_3CD). Purified $G\alpha_{13}$ bound to GST-β_3CD, but not to GST (Fig. 2C). Purified $G\alpha_{13}$ also bound to the β_1 integrin cytoplasmic domain fused with GST (GST-β_1CD) (Fig. 2D). The binding of $G\alpha_{13}$ to GST-β_3CD and GST-β_1CD was detected with GDP-loaded $G\alpha_{13}$, but enhanced by GTP-γS and AlF_4^- (Fig. 2C, 2D), indicating that the cytoplasmic domains of β_3 and β_1 can directly interact with $G\alpha_{13}$, and GTP enhances the interaction. The $G\alpha_{13}$-β_3 interaction was enhanced in platelets adherent to fibrinogen, and by thrombin, which stimulates GTP binding to $G\alpha_{13}$ via GPCR (Fig. 2E). Hence, the interaction is regulated by both integrin occupancy and GPCR signaling.

To map the β_3 binding site in $G\alpha_{13}$, we incubated cell lysates containing Flag-tagged wild type or truncation mutants of $G\alpha_{13}$ (Fig. S5) with GST-β_3CD beads. GST-β_3CD associated with wild type $G\alpha_{13}$ and the $G\alpha_{13}$ 1-212 fragment containing α helical region and switch region I (SRI), but not with the $G\alpha_{13}$ fragment containing residues 1-196 lacking SRI (Fig. 2F). Thus,

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SRI appears to be critical for β_3 binding. To further determine the importance of SRI, $G\alpha_{13}$-β_3 binding was assessed in the presence of a myristoylated synthetic peptide, Myr-LLARRPTKGIHEY (mSRI), corresponding to the SRI sequence of $G\alpha_{13}$ (197-209) (21). The mSRI peptide, but not a myristoylated scrambled peptide, inhibited $G\alpha_{13}$ binding to β_3 (Fig. 2G), indicating that mSRI is an effective inhibitor of β_3-$G\alpha_{13}$ interaction. Therefore, we further examined whether mSRI might inhibit integrin signaling. Treatment of platelets with mSRI inhibited integrin-dependent phosphorylation of c-Src Tyr^{416} and accelerated RhoA activation (Fig. 3A). The effect of mSRI is unlikely to result from its inhibitory effect on the binding of RhoGEFs to $G\alpha_{13}$ SRI because $G\alpha_{13}$ binding to RhoGEFs stimulates RhoA activation, which should be inhibited rather than promoted by mSRI (21). Thus, these data suggest that β_3-$G\alpha_{13}$ interaction mediates activation of c-Src and inhibition of RhoA. Furthermore, mSRI inhibited integrin-mediated platelet spreading (Fig. 3B), and this inhibitory effect was reversed by C3 toxin (which catalyzes the ADP ribosylation of RhoA) or Y27632, confirming the importance of $G\alpha_{13}$-dependent inhibition of RhoA in platelet spreading. Thrombin promotes platelet spreading, which requires cdc42/Rac pathways (22). However, thrombin-promoted platelet spreading was also abolished by mSRI (Fig. 3B), indicating the importance of $G\alpha_{13}$-β_3 interaction. Thus, $G\alpha_{13}$-integrin interaction appears to be a mechanism that mediates integrin signaling to c-Src and RhoA, thus regulating cell spreading.

To further determine whether $G\alpha_{13}$ mediates inhibition of integrin-induced RhoA-dependent contractile signaling, we investigated the effects of mSRI and depletion of $G\alpha_{13}$ on platelet-dependent clot retraction (shrinking and consolidation of a blood clot requires integrin-dependent retraction of platelets from within) (7,8). Clot retraction was accelerated by mSRI and depletion of $G\alpha_{13}$ (Fig. 4, A and B,Fig. S6), indicating that $G\alpha_{13}$ negatively regulates RhoA-dependent platelet retraction and coordinates cell spreading and retraction. The coordinated cell spreading-retraction process is also important in wound healing, cell migration and proliferation (23).

The function of $G\alpha_{13}$ in mediating the integrin-dependent inhibition of RhoA contrasts with the traditional role of $G\alpha_{13}$, which is to mediate GPCR-induced activation of RhoA. However, GPCR-mediated activation of RhoA is transient, peaking at 1 minute after exposure of platelets to thrombin, indicating the presence of a negative regulatory signal (Fig. 4, D and F). Furthermore, thrombin-stimulated activation of RhoA occurs during platelet shape change before substantial ligand binding to integrins (Fig. 4, C, D and F). In contrast, following thrombin stimulation, β_3 binding to $G\alpha_{13}$ was diminished at 1 minute when $G\alpha_{13}$-dependent activation of RhoA occurs, but increased after the occurrence of integrin-dependent platelet aggregation (Fig. 4, E and F). Thrombin-stimulated binding of $G\alpha_{13}$ to $\alpha_{IIb}\beta_3$ and simultaneous RhoA inhibition both require ligand occupancy of $\alpha_{IIb}\beta_3$, and are inhibited by the integrin inhibitor RGDS (Fig. 4, D-F). Thus, our study demonstrates not only a function of integrin $\alpha_{IIb}\beta_3$ as a non-canonical $G\alpha_{13}$-coupled receptor but also a new concept of $G\alpha_{13}$-dependent dynamic regulation of RhoA, in which $G\alpha_{13}$ mediates initial GPCR-induced RhoA activation and subsequent integrin-dependent RhoA inhibition (Fig. 4G). These findings are important for our understanding on how cells spread, retract, migrate, and proliferate, which is fundamental to development, cancer, immunity, wound healing, hemostasis and thrombosis.

Supplementary Material

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Acknowledgments

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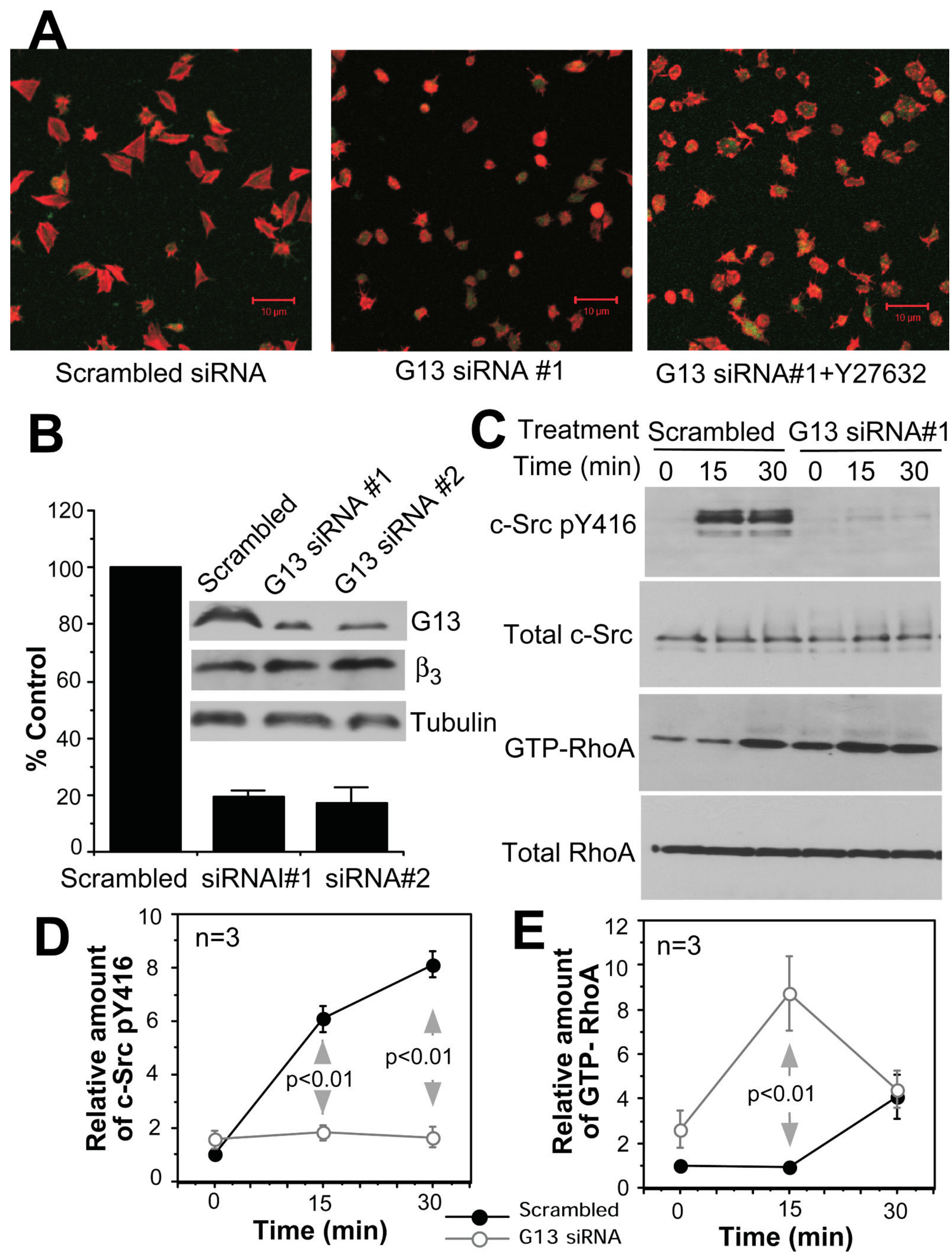

Fig. 1. The role of Gα_{13} in integrin outside-in signaling

(A) Confocal microscopy images of spreading scrambled siRNA control platelets or Gα_{13}-depleted platelets (Gα_{13}-siRNA) on fibrinogen, without or with Y27632. Merged EGFP (green) fluorescence and Alex Fluor 546-conjugated phalloidin (Red) fluorescence. (B) Western blot comparison of Gα_{13} abundance in platelets from mice innoculated with control siRNA- or Gα_{13}-siRNA-transfected bone marrow stem cells. (C, D, E) Mouse platelets from scrambled siRNA- or Gα_{13} siRNA-transfected stem cells were allowed to adhere to immobilized fibrinogen, solubilized and analyzed for c-Src Tyr^{416} phosphorylation and RhoA activation.

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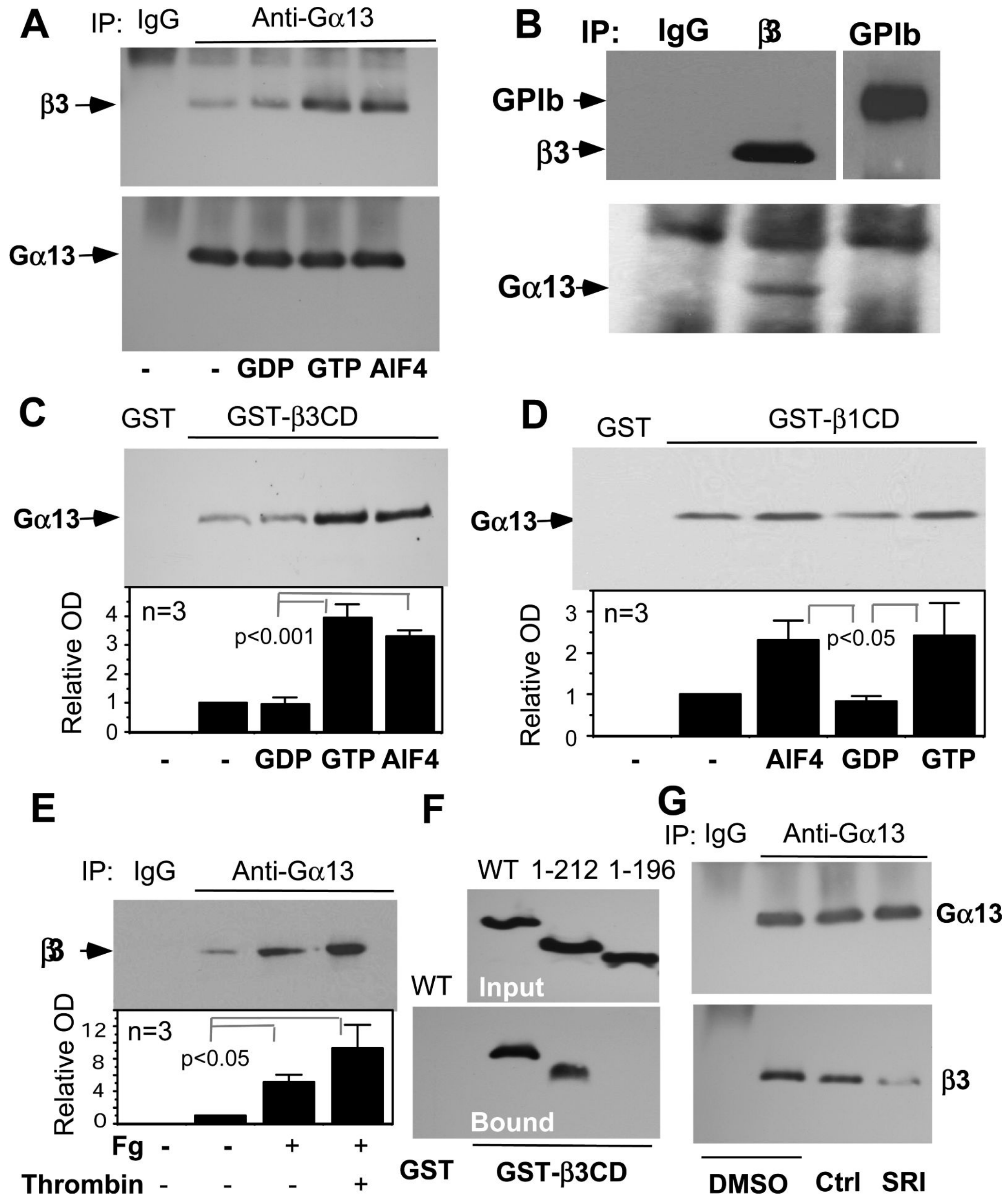

Fig. 2. Binding of $G\alpha_{13}$ to β_3 and the inhibitory effect of mSRI peptide

(A) Proteins from platelet lysates were immunoprecipitated with control IgG or antibody to $G\alpha_{13}$ with or without 1 μM GDP, 1 μM GTP or 30 μM AlF_4^-. Immunoprecipitates were immunoblotted with anti-$G\alpha_{13}$ or anti-β_3 (MAb15). See Fig. S4 for quantitation. (B) Proteins from platelet lysates were immunoprecipitated with control mouse IgG, anti-$\alpha_{IIb}\beta_3$ (D57 (24)) or an antibody to the glycoprotein Ibα (GPIb). Immunoprecipitates were immunoblotted with anti-$G\alpha_{13}$, anti-β_3, or anti-GPIb antibodies. (C, D) Purified GST-β3CD (C) or GST-β1CD (D) bound to glutathione beads was mixed with purified $G\alpha_{13}$ with or without 1 μM GDP, 1 μM GTPγS or 30 μM AlF_4^-. Bound proteins were immunoblotted with anti-$G\alpha_{13}$. Quantitative data are shown as mean±SD and p value (t-test). (E) Lysates of control platelets or platelets adherent to fibrinogen in the absence or presence of 0.025 U/ml thrombin were immunoprecipitated with anti-$G\alpha_{13}$, and then immunoblotted with MAb15. Quantitative data are shown as mean±SD and p value (t-test). (F) Lysates from 293FT cells transfected with

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Flag-tagged wild type Gα_{13} or indicated truncation mutants (see Fig. S5) were precipitated with GST-β_3CD- or GST-bound glutathione beads. Bead-bound proteins were immunoblotted with anti-Flag (Bound). Flag-tagged protein amounts in lysates are shown by anti-Flag immunoblot (Input). (G) Protein from platelet lysates treated with 0.1% DMSO, 250 μM scrambled control peptide (Ctrl) or mSRI were immunoprecipitated with anti-Gα_{13}. Immunoprecipitates were immunoblotted with anti-Gα_{13} or anti-β_3. See Fig. S4 for quantitation.

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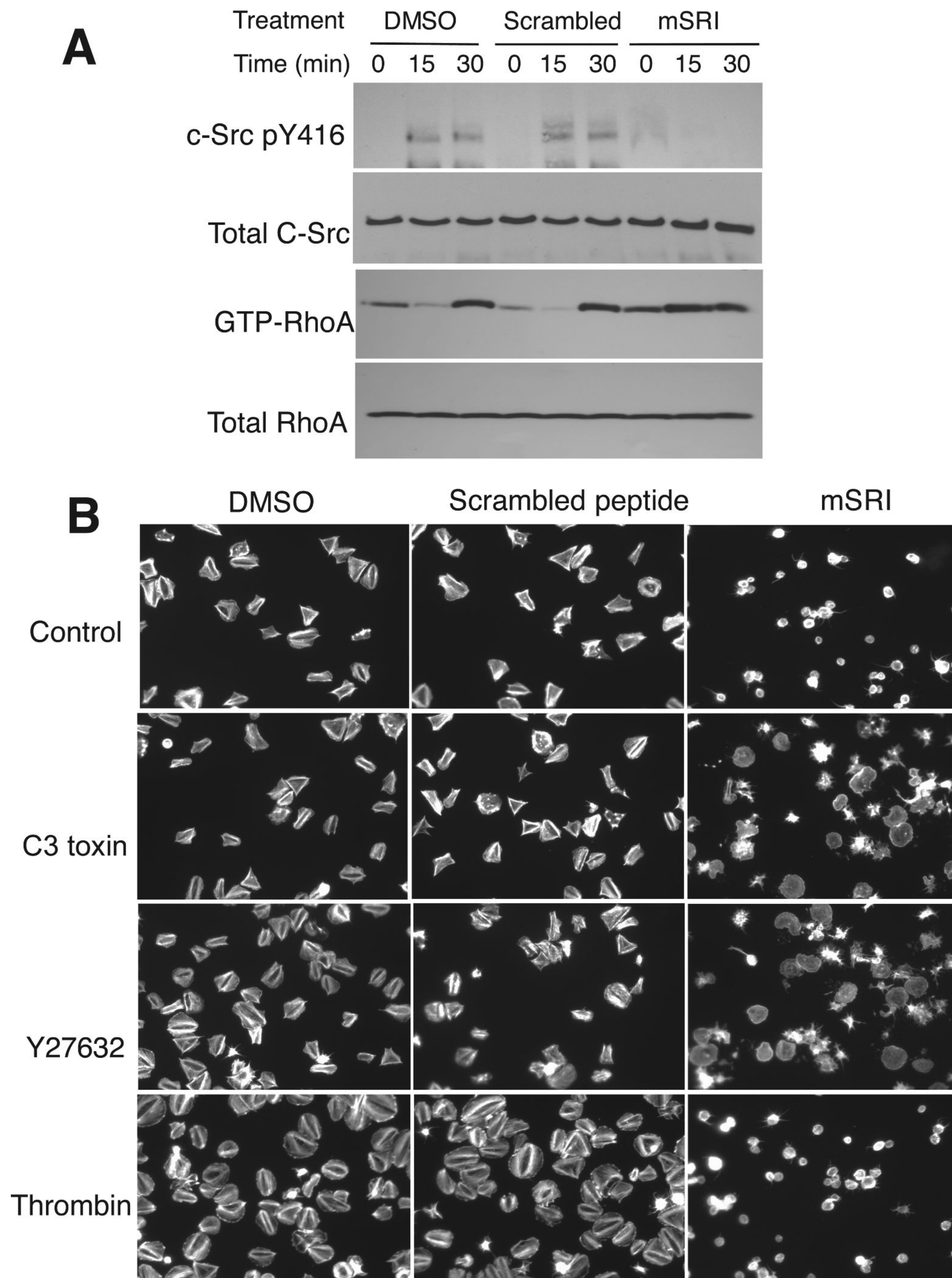

Fig. 3. Effects of mSRI on integrin-induced c-Src phosphorylation, RhoA activity and platelet spreading

(A) Washed human platelets pre-treated with DMSO, mSRI, or scrambled control peptide were allowed to adhere to fibrinogen and then solubilized at indicated time points. Proteins from lysates were immunoblotted with antibodies to c-Src phosphorylated at Tyr^{416}, c-Src, or RhoA. GTP-bound RhoA was measured by association with GST-RBD beads (25). See Fig. S4 for quantitative data. (B) Spreading of platelets treated with 0.1% DMSO, scrambled control peptide, or mSRI, in the absence or presence of C3 toxin, Y27632, or 0.01 U/ml thrombin. Platelets were stained with Alexa Fluor 546–conjugated phalloidin.

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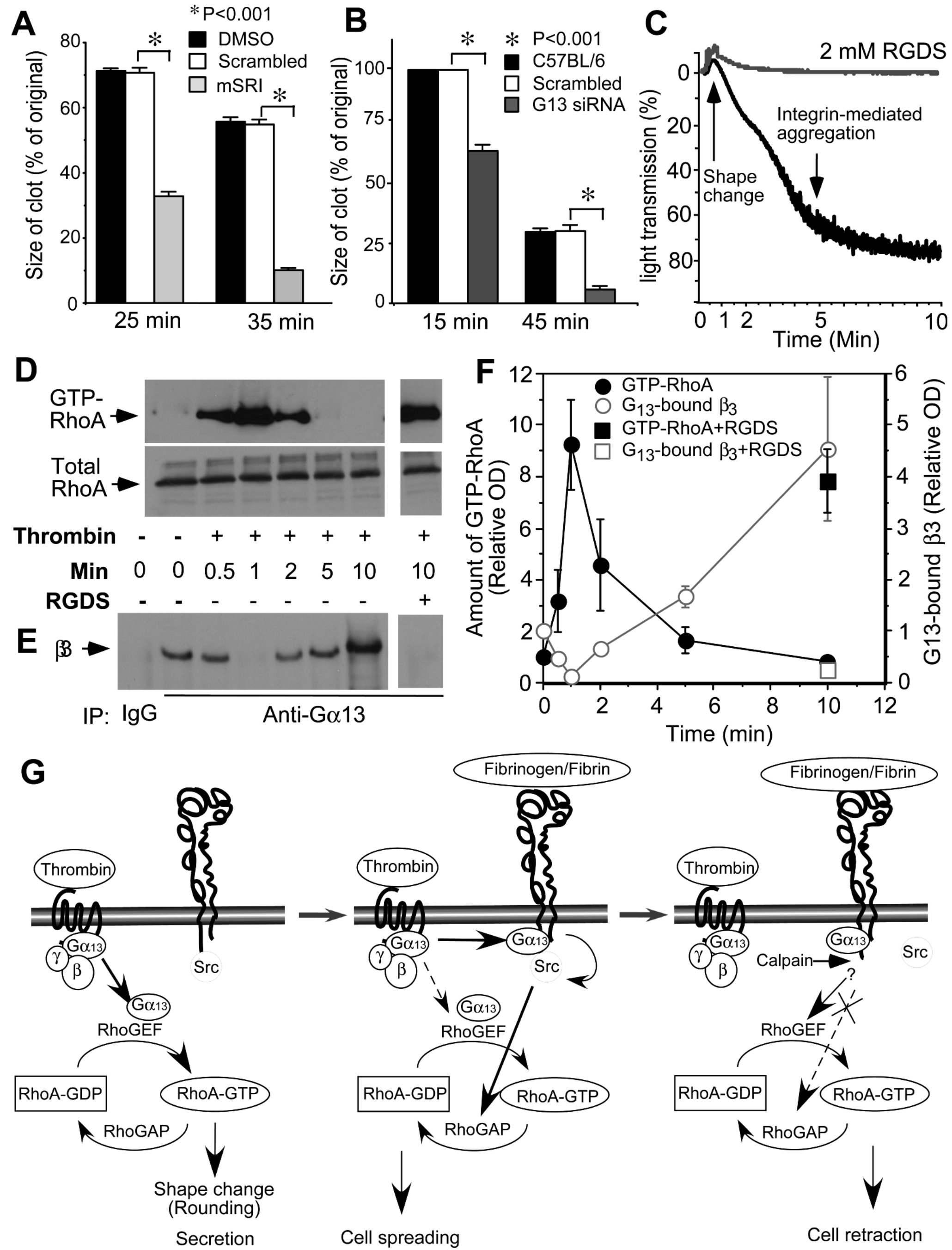

Fig. 4. The role of $G\alpha_{13}$ in clot retraction and dynamic RhoA regulation

(A) Effect of 250 μM mSRI peptide on clot retraction of human platelet-rich plasma compared with DMSO and scrambled peptide. Clot sizes were quantified using Image J (mean±SD, n=3, t-test). (B) Comparison of clot retraction (mean±SD, n=3, t-test) mediated by control siRNA platelets and $G\alpha_{13}$-depleted platelets. (C, D, E, F) Platelets were stimulated with thrombin with or without 2 mM RGDS, and monitored for turbidity changes of platelet suspension caused by shape change and aggregation (C). The platelets were then solubilized at indicated time points and analyzed for amount of β_3 coimmunoprecipitated with $G\alpha_{13}$ (D) and amount of GTP-RhoA bound to GSTRBD beads (E) by immunoblot. (F) quantitative data (mean±SD) from 3

experiments. (G) A schematic illustrating Gα_{13}-dependent dynamic regulation of RhoA and crosstalk between GPCR and integrin signaling.

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