Identification of Shc as the Primary Protein Binding to the Tyrosine-phosphorylated β3 Subunit of αIIbβ3 during Outside-in Integrin Platelet Signaling*

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Outside-in signaling mediated by the integrin αIIbβ3 (GPIIbIIIa) is critical to platelet function and has been shown to involve the phosphorylation of tyrosine residues on the cytoplasmic tail of β3. To identify proteins that bind directly to phosphorylated β3, we utilized an affinity column consisting of a peptide modeled on the tyrosine-phosphorylated cytoplasmic domain of β3. Tandem mass spectrometric sequencing and immunoblotting demonstrated that Shc was the primary protein binding to phosphorylated β3. To determine the involvement of Shc in outside-in αIIbβ3 signaling, the phosphorylation of Shc during platelet aggregation was examined; transient Shc phosphorylation was observed when thrombin-stimulated platelets were allowed to aggregate or when aggregation was induced by an LIBS (ligand-induced binding site) antibody, D3. Moreover, Shc was co-immunoprecipitated with tyrosine-phosphorylated β3 in detergent lysates of aggregated platelets. Using purified, recombinant protein, it was found that the binding of Shc to monophosphorylated (C-terminal tyrosine-phosphorylated cytoplasmic domain of Shc) and diphosphorylated β3 peptides was direct, demonstrating Shc recognition motifs on phospho-β3. Aggregation-induced Shc phosphorylation was also observed to be robust in platelets from wild-type mice, but not in those from mice expressing (Y747F,Y759F) β3, which are defective in outside-in αIIbβ3 signaling. Thus, Shc is the primary downstream signaling partner of β3 in its tyrosine phosphorylation outside-in signaling pathway.

Integrins are a homologous family of receptors that function to mediate cell adhesions and to signal cell activities such as differentiation, proliferation, and migration (1, 2). αIIbβ3 is the most prominent platelet integrin, and is capable of binding several adhesive proteins, including fibrinogen and von Willebrand factor, the binding of which mediates platelet aggregation (3). During platelet aggregation, αIIbβ3 is also involved in transmitting signals, so-called “outside-in” αIIbβ3 signaling. Outside-in αIIbβ3 signaling initiates subsequent platelet responses, including various signaling reactions, changes in the platelet cytoskeleton, and platelet secretion (4–6). The high degree of homology within the integrin family, for example between β3 and β1, and the presence of the β3 subunit in the widely distributed α,β3 integrin, suggest that αIIbβ3 signaling serves as a prototype for the elucidation of signaling pathways within the integrin family of adhesion receptors.

Outside-in αIIbβ3 signaling in platelets has been shown to involve the integrin cytoplasmic tyrosine (ICY)1 domain of the cytoplasmic tail of β3 (7). The β3 ICY domain contains two tyrosine sequences, each in an NXXY motif, separated by 11 amino acids. Phosphorylated NXXY motifs in other receptors are known to be recognition sequences for proteins containing phosphotyrosine binding (PTB) domains (8). Phosphorylation of the ICY domain of β3 occurs during outside-in signaling induced by platelet aggregation, independent of the platelet agonist (9, 10). The importance of phosphorylation of ICY domain tyrosines has been established through the analysis of platelet function in the diYF mouse, a mutant strain in which the endogenous β3 gene was replaced by one in which the two cytoplasmic tyrosines in β3 (Tyr-747 and Tyr-759) were mutated to phenylalanines. Platelets from the diYF mouse aggregate poorly to low concentrations of thrombin, aggregate reversibly to ADP, and are defective in retracting clots. DiYF mice demonstrate unstable hemostasis, perhaps because of the reversible nature of their platelet aggregates. Given the importance of tyrosine phosphorylation to outside-in αIIbβ3 signaling, characterization of the binding partner(s) of tyrosine-phosphorylated β3 is essential for characterizing the molecular features of this αIIbβ3-mediated signaling event. Moreover, because conserved ICY domain sequences are found in the β subunits of several integrins, including β1, β3, β6, and β7, and because tyrosine phosphorylation appears to be involved in the migration of cells on β1 integrins, it is anticipated that information concerning the mechanisms of signaling through αIIbβ3 might be applicable to other integrins.

Previous studies have identified proteins that bind the cytoplasmic tails of αIIbβ3. These proteins include; β3 endonexin (11), which specifically interacts with the NITY motif at residues 756–759 on the distal end of the β3 cytoplasmic tail (12), in a phosphorylation-independent manner; calcium- and integrin-binding protein (13), which binds in a calcium-dependent manner to the cytoplasmic tail of the αIIb subunit (14); talin (15), a cytoskeletal protein that binds directly to the cytoplasmic tail of both αIIbβ3 subunits, integrin-associated protein (16), a protein that laterally associates with αIIbβ3 and activates this integrin in response to thrombospondin (17); and myosin, Shc, and Grb2, which have previously been shown to bind tyrosine-phosphorylated β3 peptides (9, 10).

In the present study we used affinity chromatography and

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1 The abbreviations used are: ICY, integrin cytoplasmic tyrosine; PTB, phosphotyrosine binding; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; ERK, extracellular signal-regulated kinase; diP, doubly phosphorylated β3 peptide.
tandem mass spectrometric sequencing to identify the major soluble platelet protein binding to a diphosphorylated β3 ICY domain cytoplasmic tail peptide. Biochemical and genetic strategies were then used to determine whether the protein identified was involved in outside-in signaling through αIIbβ3. We discovered that Shc was the primary protein associating with the phosphorylated β3 peptide and showed that Shc becomes transiently phosphorylated during platelet aggregation and can be co-immunoprecipitated with β3 from lysates in which outside-in αIIbβ3 signaling occurs. We also found that Shc interacts directly with tyrosine-phosphorylated β3, because a GST-p52She fusion protein binds directly to the diphosphorylated β3 peptide sequence and to the β3 peptide sequence monophosphorylated on the C-terminal end. Analysis of platelets from the diYF mouse expressing (Y747F,Y759F) β3 showed that, in platelets where β3 tyrosine phosphorylation cannot occur and where outside-in αIIbβ3 Shc phosphorylation is defective, the level of aggregation-induced Shc phosphorylation was abrogated. These data implicate Shc as playing a major role in outside-in αIIbβ3 signaling in platelets by serving as a direct signaling partner for tyrosine-phosphorylated β3.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Horseradish peroxidase-conjugated Protein A (NA 9120) and ECL Western blotting detection reagents (RPN 2106) were from Amersham Pharmacia Biotech; biotin-conjugated anti-rabbit IgG controls (sc-2040) and horseradish peroxidase-conjugated anti-mouse GST monoclonal antibody (sc-138 HRP) were from Santa Cruz Biotechnologies; anti-rabbit Shc polyclonal antibodies (sc-695, s14630) were from Santa Cruz Biotechnologies and Transduction Laboratories, respectively; anti-Shc antibody sc-1695 was custom biotinylated at Santa Cruz Biotechnologies and Transduction Laboratories, respectively; anti-mouse phosphotyrosine antibodies PY-20 (P11120) and 4G10 (55-321) were from Transduction Laboratories and Upstate Biotechnology, respectively. The rabbit polyclonal antibody against αIIbβ3, #4518 was from COR Therapeutics, Inc. The anti-LIBS antibodies D3, was the kind gift of Dr. Lisa K. Jennings (University of Tennessee, Memphis). Peptides were synthesized at SynPep Corp. utilizing solid-phase Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry, except for the diphosphorylated, scrambled β3 peptide, and the β3 peptide monophosphorylated on the C-terminal tyrosine residue, both synthesized at Research Genetics. E. coli restriction enzymes were from Novagen. GST and GST-Shc fusion proteins were then expressed in and purified from E. coli. For the expression and purification of recombinant Shc, the pGEX-3X bacterial expression vector (for expression of GST-p52She fusion protein in E. coli), was a kind gift from Drs. Darren Tyson and Ralph A. Bradshaw, University of California at Irvine. Precast SDS gels were from Bio-Rad.

**Affinity Chromatography Procedure**—For the column preparation, immobilized avidin on 6% cross-linked beaded agarose (1 mg/ml) was pre-equilibrated in 1:1 TBS/lysis buffer A (10 mM Tris base, pH 7.4, 75 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na3VO4, 1 mM PMSF, and 10 μg/ml of both aprotinin and leupeptin), then incubated 12 h (overnight) with the biotinylated β3 peptide (biotin-DTTNPpYKEATSTFTNITYRGT-COOH) or with the diphosphorylated β3 peptide (biotin-DTTNPpYKEATSTFTNITYRGT-COOH). Excess peptide was drained off the column material through 1.5-12-cm polypropylene columns (Bio-Rad) by gravity filtration. All three types of columns to be used in the purification process, i.e. 20-ml column beds of avidin-agarose, non-phosphorylated, biotin-β3 conjugated to avidin-agarose, or diphosphorylated biotin-β3 conjugated to avidin-agarose, were equilibrated in 1:1 TBS/lysis buffer A. Columns were set up in series, with avidin-agarose as the first, pre-clear column, followed by the non-phosphorylated β3 column, followed by the diphosphorylated β3 column. Human platelets were obtained from volunteers subjected to double platelet pheresis, conducted at the Stanford Blood Center, Palo Alto, CA and used within 2 h of collection. Approximately 5 × 1011 platelets were obtained from each donor. Platelet lysates (13 mM sodium citrate, 30 mM glucose, 120 mM NaCl, pH 7.0) and resuspended in 210 ml of calcium/magnesium free Tyrode’s Heps buffer (12 mM NaHCO3, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, pH 7.4). Platelet suspensions (2.95 ± 0.02 platelets/ml, total volume 170 ± 20 ml) were lysed on ice for 30 min with a 1:1 volume of 2× lysis buffer A. Lysates were centrifuged at 100,000 × g for 1 h at 4 °C to remove the majority of cytoskeletal proteins. The supernatants were then loaded at a rate of 20 ml/h onto the columns set up in series. Columns were then separated, washed individually with 8 bed volumes of 1:1 TBS/lysis buffer A, pH 7.4, and eluted with 0.8 mM phenyl phosphate. Samples (12 μl) from fractions were monitored by silver staining of SDS gels and by Western blotting using phosphotyrosine antibodies. Fractions containing a phosphotyrosine kinase activity were detected by silver staining and eluting exclusively off the diphosphorylated β3 column were pooled, concentrated by centrifugal ultrafiltration (Millipore), precipitated (18), and run on SDS gels. Bands of interest were excised from SDS gels and sequenced by tandem mass spectrometry at the Harvard Microchemistry Facility (Dr. William S. Lane).

**Preparation of Human Platelet Lysates and Immunoprecipitation of Shc**—Blood was drawn, and washed platelets were prepared as in a previous study (19) with the addition of 50 ng/ml prostaglandin I2 and 0.6 unit/ml arylsulfatase in the collecting solution. The platelet pellet was resuspended in Tyrode’s Hepes buffer, pH 7.4, 1 mM CaCl2, and 1 mM MgCl2, to a concentration of 7 × 107 platelets/ml, and eluted with 0.8M slurry of avidin-agarose (in PBS, pH 7.4). At the end of the “pre-clear” steps, lysates were centrifuged at 14,000 × g for 10 min at 4 °C. Samples were split, and 2 μg of either biotinylated anti-Shc or biotinylated rabbit control IgG was added to each 500-μl sample. Incubations with the antibodies continued overnight at 4 °C. Samples were then incubated with 70 μl of 50% avidin-agarose bead slurry for 1 h at 4 °C. Immunoprecipitation reactions were spun 20 s at 14,000 × g, the supernatant was aspirated off, and the pellet was washed with 250 μl of 0.5 M NaCl in 1× lysis buffer B. Following centrifugation, the wash steps were repeated twice using 1× lysis buffer B. The remaining pelleted beads were resuspended in 2× reducing sample buffer, boiled, and subjected to SDS-PAGE gel electrophoresis.

**Western Blotting Analysis**—Samples from immunoprecipitations and from peptide precipitations of purified recombinant Shc were run on gels, which were then transferred to 0.2-μm nitrocellulose supports and blotted with specific antibodies. For the immunoprecipitation experiments, blots of samples were cut in half and probed for phosphorylated tyrosine residues (using PY20 and 4G10) and αIIbβ3 (4518); blots probed with antibodies to platelet-specific phosphotyrosine epitopes were reprobed with Shc antibodies. These blot of samples from immunoprecipitations using biotinylated antibodies were developed with Protein A conjugated to horseradish peroxidase and detected with ECL Western blotting detection reagents. Thus, IgG heavy chain of the immunoprecipitating, biotinylated anti-rabbit Shc polyclonal and anti-rabbit IgG control antibodies was not detected, and the observed band patterns were not obscured. Blots of IgG control immunoprecipitations showed no co-immunoprecipitation of tyrosine-phosphorylated proteins (data not shown).

**Expression and Purification of Recombinant Shc**—The pGEX-3X bacterial expression plasmid and the pGEX-3X-p52She construct (20), the latter which contained the p52 Shc sequence inserted in-frame with the pGEX-3X glutathione S-transferase (GST) sequence at the unique EcoRI site, were used to transform the protease-deficient BL21(DE3)pLysS E. coli strain of cells (Novagen). For GST and GST-Shc fusion protein expression, bacterial preparations were induced by 1 mM isopropyl-1-thio-galactopyranoside at 37 °C for 2 h. Bacteria were lysed in 2× lysis buffer B (2% Triton X-100, 1 mM dithiothreitol, 8 mM Na3VO4, 2 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml aprogin). After centrifugation at 20,000 × g for 1 h, 4°C, the expressed GST and GST fusion proteins were isolated from bacterial cell lysates by affinity chromatography using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), which were pre-equilibrated in 2× lysis buffer B. GST and GST-Shc fusion proteins were then eluted with reduced glutathione (10 mM).

**Analysis of the Interaction of Recombinant Shc with β3**—To deter-
amine whether Shc can bind to phosphorylated β3, equal amounts of GST and GST-Shc fusion proteins were mixed with one of non-phosphorylated, monophosphorylated (on the N-terminal tyrosine; biotin-DTAN-NPLpYKEATSTFTNITYRG7-COOH), monophosphorylated (on the C-terminal tyrosine; biotin-DTANPLpYKEATSTFTNPYYRG7-COOH), scrambled diphosphorylated (biotin-TNlNEARtYASKLDPG7TTPYlTNN), or diphosphorylated β3, peptide, 50 μg of which was bound to avidin-agarose and suspended in 1× lysis buffer B. After incubation for 2 h at 4 °C, the avidin-agarose-bound phospho or non-phospho β3 pep-
tides were washed with 1× lysis buffer B. Beads were resuspended in 2× sample buffer, and samples were separated on a 4–15% SDS-polyacrylamide gel. Gels were blotted onto nitrocellulose membranes and probed for the presence of Shc and GST with corresponding polyclonal antibodies (Santa Cruz Biotechnology).

Preparation of Mouse Platelet Lysates and Immunoprecipitation of Shc—The Shc immunoprecipitation procedure described above was optimized for mouse blood. First, blood was obtained by cardiac puncture (700 μl) and drawn into a mix of 140 μl of 2.5% trisodium citrate/2% dextrose/1.5% citric acid monohydrate, 560 μl of saline, and protaglandin E1, at 50 ng/ml final concentration. Washed platelets were obtained as described (21). Platelets pooled from five to six animals were resuspended in Tyrode’s buffer with 1 mM MgCl2, at 6 × 10^9/ml. Thrombin-induced platelet aggregation was measured in the aggregometer. Platelets were lysed 15 s after thrombin by the addition of an equal volume of 2× lysis buffer B into the aggregometer tube. Samples were then incubated on ice for 20 min prior to sonication (6 × 10 s bursts at amplitude 60). Lysates were preclarified with 50 μl of avidin-agarose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C then spun in a microcentrifuge at 16,000 × g for 15 min at 4 °C. Supernatants were recovered, and for each immunoprecipitate, 200 μl of lyase was incubated with 2 μg of either biotinylated rabbit IgG (control) or biotinylated anti-Shc antibody and 50 μl of avidin-agarose overnight at 4 °C. Beads were washed and resuspended in sample buffer, as with human immunoprecipitates, and loaded onto SDS gels for Western blotting analysis.

RESULTS

Purification and Identification of Proteins that Bind to Diphosphorylated β3 Peptides—To identify platelet proteins that selectively bind the phosphorylated tail of β3, we designed an affinity procedure that had three columns in series: avidin-agarose, the support matrix; a protein that was not found in eluates of either the avidin-agarose precolumn or the only Shc isoform found in platelets.

Shc Protein Binding to the β3 Subunit of αIIbβ3

Fig. 1. Identification of Shc as the major protein specifically eluting from a diphosphorylated β3 peptide column. Platelets were lysed in 1% Nonidet P-40 lysis buffer, and the majority of cytoskeletal proteins were removed by ultracentrifugation. The supernatant was run over three columns in series; Pre, avidin-agarose precolumn; β3, the non-phosphorylated β3 peptide column; diP, the doubly phosphorylated β3 peptide column. Columns were washed separately, and proteins were eluted using 0.8 M phenyl phosphate. Samples from fractions eluted off of all three columns were run on 4–15% SDS-polyacrylamide gels and silver-stained. The gel shown here represents fractions 17 through 20 and is representative of three independent experiments. The arrow indicates the migration position of a band that specifically eluted from the diphosphorylated β3 column.

Ssh is Tyrosine-phosphorylated during Thrombin-induced Aggregation—To determine whether Shc is involved in outside-in signaling through αIIbβ3, we determined the time course of Shc tyrosine phosphorylation during platelet aggregation, an event known to induce αIIbβ3 outside-in signaling. Fig. 4 shows that phosphorylation during platelet aggregation induced by thrombin, a potent platelet agonist. Shc tyrosine phosphorylation increased dramatically during thrombin-induced aggregation (11.4 ± 0.8-fold), reaching a maximum 20 s after the addition of thrombin. With continued stirring, the level of tyrosine phosphorylation decreased, to 9.1 ± 0.5-fold at 45 s, to 6.8 ± 0.4-fold at 90 s of aggregation, and to 1.4 ± 0.1-fold at 270 s. A reprobe of the immunoprecipitate blot with Shc polyclonal antibodies showed that each lane contained an equal amount of Shc (middle panel). Thrombin signaling alone, in the absence of aggregation, is sufficient to induce tyrosine phosphorylation of several platelet proteins (22, 23). To determine the extent of Shc phosphorylation due to thrombin activation as compared with thrombin aggregation, the αIIbβ3-specific antagonist epifibatide was used to prevent aggregation. Although Shc phosphorylation still occurred in the absence of aggregation, the -fold increase was approximately half of that observed when aggregation also occurred, 6.2 ± 0.3 at 20 s, 4.3 ± 0.4 at 45 s, and to control levels at 270 s. Thus, Shc tyrosine phosphorylation, much like that observed with Syk, appears to be induced by two pathways in platelets: one induced by thrombin stimulation, most likely a pathway initiated by protease-activated receptor activation and involving inside-out αIIbβ3 signaling; and the other induced by platelet aggregation, most likely a pathway initiated by αIIbβ3 outside-in signaling.

Previous studies have shown that outside-in signaling in-
Shc Protein Binding to the β₃ Subunit of αIIBβ₃

Fig. 3. Western blotting analysis of samples eluted from the diphospho-β₃ column. Fractions 1 through 39 eluted from the β₃ diP column using phenyl phosphate, were subjected to gel electrophoresis. The separated proteins were then transferred to nitrocellulose and subjected to immunoblotting with an anti-Shc antibody. Shc was detected in most of the fractions with peak elution being observed in fractions 19 through 24.

Recombinant Shc Binds to Phospho-β₃ Peptides—The co-immunoprecipitation of Shc with β₃ following platelet aggregation suggests that Shc either binds directly to the tyrosine-phosphorylated ICY domain of β₃ or to other phospho-β₃ binding proteins. To determine whether the tyrosine-phosphorylated ICY domain of β₃ has Shc recognition motifs, a GST-p52Shc fusion protein was expressed, purified, and studied for its ability to interact with the β₃ ICY domain peptides. As depicted in Fig. 6, the GST-p52Shc fusion protein could be detected in peptide precipitates with the diphosphorylated and the C-terminal monophosphorylated β₃ peptide using both anti-GST (Fig. 6A) and anti-Shc (Fig. 6B) antibodies. Little, if any, GST-Shc was present in precipitates obtained using the unphosphorylated β₃ peptide, the β₃ peptide monophosphorylated at the N terminus, and the diphosphorylated β₃ scramble peptide. Fig. 6C depicts the binding capacity of the C-terminal monophosphorylated and the diphosphorylated β₃ peptides for the purified Shc fusion protein. The diphosphorylated β₃ peptide has a significantly greater binding capacity for Shc than the monophosphorylated peptide. Thus, Shc binds the ICY domain of β₃, but only when this domain is phosphorylated on β₃ tyrosine residue 759.

Tyrosine Phosphorylation of Shc in Marine Platelets—As a genetic test of the hypothesis that phospho-Shc is the downstream component of β₃ tyrosine phosphorylation during outside-in signaling, we assessed the level of Shc phosphorylation in platelets from the diYF mouse, where the native β₃ gene is replaced by one lacking cytoplasmic tyrosines (Y747F, Y759F) β₃). Wild-type and diYF platelets were aggregated using a high

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Shc Protein Binding to the $\beta_3$ Subunit of $\alpha_{IIb}\beta_3$

Numerous strategies have been developed to identify signaling partners of membrane receptors (reviewed in Refs. 26–29), but we elected to use affinity chromatography, because platelets can be isolated in high yield and they contain high concentrations of signaling proteins (3), and we wanted to identify the primary protein(s) that bind tyrosine-phosphorylated $\beta_3$. Previous studies have established that phosphopeptides modeled on the sequence of membrane receptors are useful for identifying signaling partners (9, 31, 32). The first of three assumptions made in the affinity approach used in the present study was that di-phosphorylated $\beta_3$ is generated during platelet aggregation. It has been established that $\beta_3$ becomes associated with the myosin-based platelet cytoskeleton following platelet aggregation (19). Because only $\beta_3$ peptides that are phosphorylated on both tyrosines bind to purified myosin (9), the data suggest that both tyrosines can become phosphorylated upon platelet aggregation. To identify as many tyrosine phosphorylation-dependent signaling partners of $\beta_3$ in platelets as possible, we therefore used the di-phosphorylated $\beta_3$ peptide in the affinity matrix. Our second assumption was that the bound, phosphorylated $\beta_3$ signaling partners would be eluted by phenyl phosphate, a reagent previously shown to disrupt interactions of anti-phosphotyrosine antibodies with tyrosine-phosphorylated proteins (33, 34). The third assumption was that proteins capable of binding to di-phosphorylated $\beta_3$ were present in detergent lysates of unstimulated platelets. This assumption is based on findings showing that phosphorylation of $\beta_3$ occurs only upon platelet aggregation (10), suggesting that prior activation of phosphotyrosine binding domains of signaling proteins was not required for binding to the phosphorylated integrins.

The affinity chromatography approach described herein identified the p52 isoform of Shc as the primary protein in human platelets that binds to the phosphorylated ICY domain of $\beta_3$. A previous study found that, of the three Shc isoforms, only p52Shc is present in platelets. In support of this, our data using Western analysis of detergent lysates and of the isolated protein detected only p52 Shc and not p46 or p66 isoforms. Also, sequence analysis of the tryptic peptides produced from the isolated Shc only revealed peptides contained within the p52 isoform of the protein.
Previous studies showed that Shc becomes tyrosine-phosphorylated upon thrombin-induced platelet aggregation and that Shc bound to diphosphorylated peptides (9). Three biochemical studies of platelets have now established that Shc is a proximal component of the phosphotyrosine $\beta_3$ signaling pathway. First, because one of the hallmarks of Shc involvement in a signaling pathway is that it becomes tyrosine-phosphorylated (reviewed in Ref. 35), our data demonstrating that tyrosine phosphorylation of Shc occurs upon LIBS-induced aggregation places Shc directly on the $\alpha_{IIb}\beta_3$ outside-in signaling pathway. LIBS antibodies are known to induce fibrinogen binding to $\alpha_{IIb}\beta_3$ by inducing a conformational change in $\alpha_{IIb}\beta_3$ without the prior requirement of activation by a platelet agonist. Thus, these data indicate that Shc phosphorylation can be specifically induced by outside-in $\alpha_{IIb}\beta_3$ signaling. Shc phosphorylation in response to thrombin differed from that observed in response to the LIBS antibody in that phosphorylation was induced upon thrombin-induced activation, with further phosphorylation occurring upon subsequent platelet aggregation.

A second hallmark of the involvement of Shc in a signaling pathway is its physical association with the cell membrane receptor responsible for initiating the signaling cascade (for example, with platelet-derived growth factor receptor or components of the B cell receptor complex). In the present study, we found that $\beta_3$ could be co-immunoprecipitated with Shc from detergent lysates of aggregated platelets but not from lysates of control, unstimulated platelets or stimulated but not aggregated platelets, demonstrating that aggregation induced an association of Shc with this integrin. Third, we have provided genetic evidence implicating Shc in the $\beta_3$ tyrosine phosphorylation signaling pathway. Platelets from the diYF mouse experiment demonstrated a direct interaction between Shc and $\beta_3$ tyrosine phosphorylation. Platelets from the diYF mouse showed direct binding of purified Shc to the diYF platelets, as shown in this study, could provide a model system for signaling through both $\beta_3$ and $\beta_5$ integrins in many different cell types.

In conclusion, we have identified Shc as the major signaling protein found in human platelets, binding exclusively to tyrosine-phosphorylated $\beta_3$, and found that Shc was important for transducing signals through $\alpha_{IIb}\beta_3$. Shc has been associated with the Ras signaling pathway through Grb2 binding to tyrosine-phosphorylated Shc (35); however, the implications for enhanced Ras signaling through $\alpha_{IIb}\beta_3$ as a result of platelet aggregation are not clear. Ras activation has been shown to occur rapidly upon thrombin and thromboxane A2 receptor stimulation in human platelets (49). And so perhaps the Ras signaling pathway is regulated by thrombin receptors during inside-out signaling, accounting for Shc phosphorylation in platelets during thrombin-induced activation. It is thought that the downstream effectors of Ras signaling, the mitogen-activated protein kinases ERK1/2, are responsible for the activation of phospholipase $A_2$ in stimulated platelets, ultimately resulting in enhanced levels of arachidonic acid. In fact, both p38 and ERK1/2 contribute to the regulation of phospholipase $A_2$ (30, 50–51). It is possible that events downstream of Shc binding to phosphorylated $\beta_3$ involve activation of the mitogen-activated protein kinase pathways, and we suggest that Shc phosphorylation through outside-in signaling operates as an important, independent pathway resulting in further arachidonic acid release and the stabilization of platelet-platelet aggregate formation. Deciphering the downstream events following Shc phosphorylation after $\alpha_{IIb}\beta_3$ integrin ligation will lead to further understanding of the mechanisms involved in platelet aggregation. The identification of Shc in integrin signaling, the high conservation of the ICY domain of $\beta_3$ within the integrin family (e.g. $\beta_1, \beta_3, \beta_6, \beta_7$), and the demonstrated role of ICY domain tyrosines in integrin function, suggest that the $\beta_3$ tyrosine phosphorylation/Shc signaling pathway may be a common feature to all forms of integrin signaling.

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