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Proximal, selective, and dynamic interactions between integrin αIIbβ3 and protein tyrosine kinases in living cells

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Stable platelet aggregation, adhesion, and spreading during hemostasis are promoted by outside-in αIIbβ3 signals that feature rapid activation of c-Src and Syk, delayed activation of FAK, and cytoskeletal reorganization. To evaluate these αIIbβ3–tyrosine kinase interactions at nanometer proximity in living cells, we monitored bioluminescence resonance energy transfer between GFP and Renilla luciferase chimeras and bimolecular fluorescence complementation between YFP half-molecule chimeras. These techniques revealed that αIIbβ3 interacts with c-Src at the periphery of nonadherent CHO cells. After plating cells on fibrinogen, complexes of αIIbβ3–c-Src, αIIbβ3–Syk, and c-Src–Syk are observed in membrane ruffles and focal complexes, and the interactions involving Syk require Src activity. In contrast, FAK interacts with αIIbβ3 and c-Src, but not with Syk, in focal complexes and adhesions. All of these interactions require the integrin β3 cytoplasmic tail. Thus, αIIbβ3 interacts proximally, if not directly, with tyrosine kinases in a coordinated, selective, and dynamic manner during sequential phases of αIIbβ3 signaling to the actin cytoskeleton.

Introduction

Ligand binding to integrins initiates "outside-in" signals that regulate cell motility and gene expression (Schwartz, 2001; Alahari et al., 2002). In platelets, the binding of fibrinogen to αIIbβ3 triggers signals that promote cytoskeletal reorganization, spreading, and aggregation (Shattil et al., 1998). Prominent biochemical events during this process include activation of the tyrosine kinases Src, Syk, FAK, and Btk and tyrosine phosphorylation of proteins in integrin-based adhesion sites (Lipfert et al., 1992; Mukhopadhyay et al., 2001; Obergfell et al., 2002). Unlike activation of FAK or Btk, activation of Src and Syk is relatively rapid and independent of actin polymerization (Clark et al., 1994; Obergfell et al., 2002). Genetic ablation or pharmacological inhibition of Src or Syk impairs platelet spreading and FAK activation, indicating that these responses are dependent on integrin activation of Src and Syk (Obergfell et al., 2002).

The molecular basis for αIIbβ3 activation of tyrosine kinases is incompletely understood. c-Src and several other Src family kinases coimmunoprecipitate with αIIbβ3 from resting platelets. In contrast, Syk coimmunoprecipitates with αIIbβ3 only after platelet adhesion to fibrinogen (Obergfell et al., 2002). Work in CHO cells indicates that c-Src and Syk activation requires αIIbβ3 clustering and an intact β3 cytoplasmic tail (Hato et al., 1998; Arias-Salgado et al., 2003). Because c-Src, Syk, and FAK can bind directly to β3 tail peptides or model β3 tail proteins in vitro (Schaller et al., 1995; Woodside et al., 2002; Arias-Salgado et al., 2003), direct interactions between β3 and tyrosine kinases may promote outside-in signaling.

To better define αIIbβ3–tyrosine kinase interactions in living cells, we have used two protein interaction reporter assays that can detect proximal (nm) interactions, bioluminescence resonance energy transfer (BRET), and bimolecular fluorescence complementation (BiFC). BRET between a Renilla luciferase (Rluc) chimera and another chimera with a luminescence acceptor, such as GFP, has been used to study homooligomerization of receptors, including integrins, by luminometry (Angers et al., 2000; Boute et al., 2002; Ramsay et al., 2002; Buensuceso et al., 2003). In BiFC, two po-

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Abbreviations used in this paper: BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; Rluc, Renilla luciferase.

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tential interacting proteins are fused to NH_2- and COOH-terminal half-molecules of YFP, respectively. If the transfected proteins interact, YFP may be reconstituted (Hu et al., 2002; Hu and Kerppola, 2003). Thus, BiFC may complement information derived from BRET by enabling visualization and subcellular localization of αIIbβ3–tyrosine kinase complexes by fluorescence microscopy. Both techniques require expression of chimeric proteins, but they have the potential to supplement biochemical and genetic analyses of integrins. Using BRET, BiFC, and proteins fused to suitable reporters, we establish here that proximal αIIbβ3–tyrosine kinase interactions occur in a coordinated and sequential manner within membrane ruffles and specific adhesion structures, providing a spatial context for outside-in αIIbβ3 signaling.

Results and discussion

Interactions of αIIbβ3 and c-Src in living cells

To evaluate the proximity between c-Src and αIIbβ3, CHO cells were transfected with β3 and chimeras of αIIb and c-Src that contain reporter groups for BRET or BiFC (Figs. 1 A and 2 A). CHO cells were used because they recapitulate outside-in αIIbβ3 signaling responses characteristic of platelets (Miranti et al., 1998; Obergfell et al., 2001). Preliminary studies with a ligand-mimetic antibody indicated that each αIIbβ3 chimera was surface expressed in a default low-affinity state, similar to wild-type αIIbβ3. Furthermore, as in platelets (Obergfell et al., 2002), c-Src and Syk chimeras became activated upon CHO cell adhesion to fibrinogen, and they coimmunoprecipitated with αIIbβ3.

To detect BRET, the Rluc and GFP reporter groups must be within ~80 nm of each other and in a favorable orientation, enabling nonradiative energy transfer between an Rluc substrate (coelenterazine) and GFP. When cells in suspension expressing αIIbRlucβ3 and either c-Src or c-SrcGFP were compared, the c-SrcGFP–expressing cells exhibited a 27.1% increase in BRET ratio ($P < 0.01$; Fig. 1 B). BRET was not affected by cell adhesion to fibrinogen (unpublished data). Minimal, nonsignificant BRET increases were observed if GFP was expressed instead of SrcGFP, or if αIIbRluc was coexpressed with β3Δ724, which lacks most of the β3 cytoplasmic tail (Fig. 1 B). Because the β3 tail interacts directly with c-Src in vitro (Arias-Salgado et al., 2003), these results are consistent with a similar direct, constitutive interaction between αIIbβ3 and c-Src in cells.

BiFC was performed by fusing αIIb to the COOH-terminal half-molecule of YFP (αIIbYC) and c-Src to the NH_2-terminal half of YFP (SrcYN; Fig. 2 A). BiFC between αIIbYCβ3 and SrcYN was observed around the periphery of nonadherent cells and at the ruffling edges of lamellipodia of fibrinogen-adherent cells, where it colocalized with antibody-labeled αIIbβ3 and c-Src (Fig. 2 B, i). In contrast, no BiFC was observed in cells expressing β3Δ724 (Fig. 2 B, ii). BiFC between αIIbYCβ3 and SrcYN also colocalized with cortactin within a distance of 3–4 μm from the leading edge (Fig. 2 C, i), and with vinculin and F-actin in small focal complexes and larger focal adhesions (Fig. 2 C, ii). Similarly, when unfixed cells were analyzed by real-time fluorescence microscopy, BiFC between αIIbYCβ3 and SrcYN was detected within membrane ruffles, focal complexes, and focal adhesions (Fig. 3 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200402064/DC1). Over a 6-min observation period, αIIbYCβ3–SrcYN complexes appeared to move from membrane ruffles to the focal adhesion structures, a conclusion supported by the predominant colocalization of the BiFC signal with known markers of these structures in fixed and stained cells (Fig. 2 C). Thus, the proximity of αIIbβ3 to c-Src and the dynamic nature of αIIbβ3–c-Src complexes upon integrin ligation provide a spatial context for initiation of outside-in αIIbβ3 signaling. Similarly, interaction between c-Src and the related integrin, αVβ3, may occur within podosomes of osteoclasts (Linder and Aepfelbacher, 2003), whereas the absence of such an interaction may help to explain the overlapping bone resorbtion defects in mice deficient in either αVβ3 or c-Src (Soriano et al., 1991; McHugh et al., 2000).

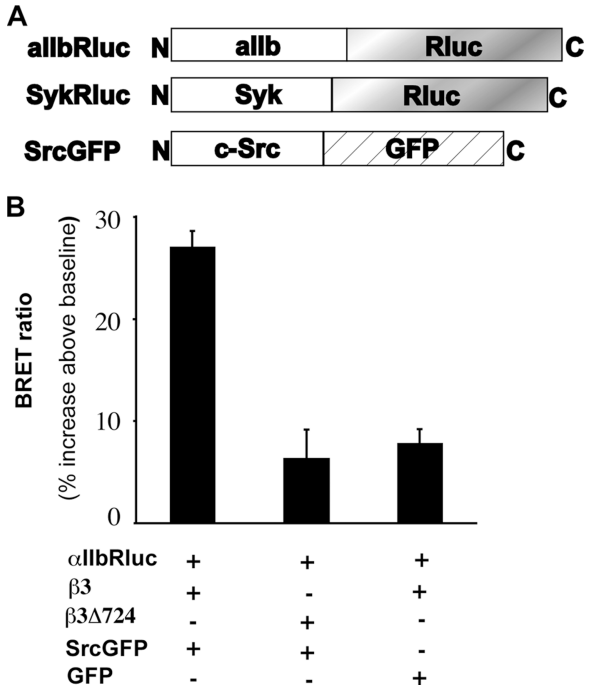

Figure 1. **Analysis of αIIbβ3–c-Src interactions by BRET.** (A) Schematic representation of the chimeras used for BRET; N, NH_2 termini; C, COOH termini. (B) αIIbβ3 and c-Src interaction in living cells. CHO cells were transiently transfected with the indicated cDNAs. After 48 h, BRET ratios were measured as described in Materials and methods. BRET ratios are depicted as the percent above a "control" baseline of zero, which was defined as the ratio in cells expressing αIIbRlucβ3 or αIIbRlucβ3Δ724 and c-Src. Data are the means ± SEM of three experiments.

Syk interacts with αIIbβ3 and c-Src, but not with FAK, in living cells

Fibrinogen binding to platelets stimulates the recruitment of Syk to an immunoprecipitable complex containing αIIbβ3 and c-Src (Obergfell et al., 2002). Consistent with this finding, an increase in BRET ratio between SykRluc and SrcGFP was observed when αIIbβ3-CHO cells were plated on fibrinogen and compared with suspension cells ($P <$ 0.01). No such increase occurred if β3Δ724 was expressed

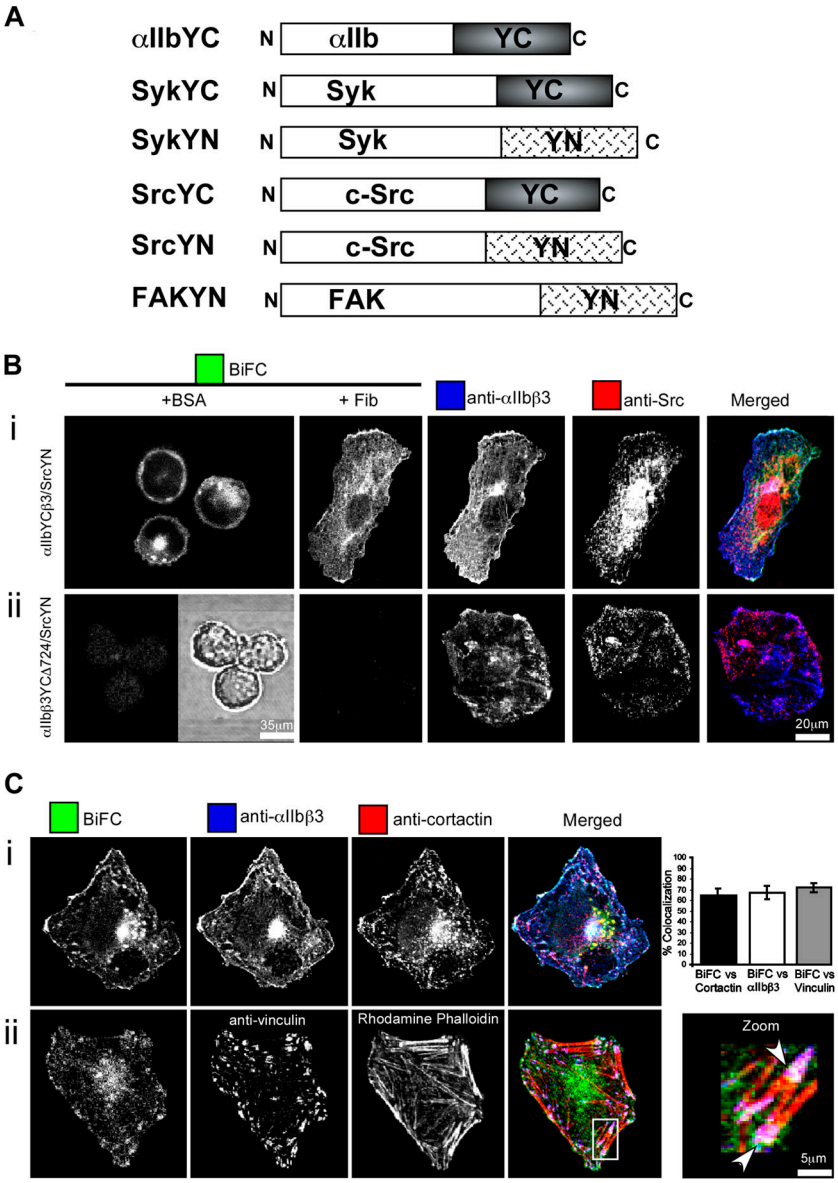

Figure 2. **Analysis of αIIbβ3–c-Src interactions by BiFC.** (A) Schematic representation of the chimeras used for BiFC analysis. (B) After transient transfection of the indicated cDNAs, cells were maintained in suspension over BSA or plated on fibrinogen (Fib) for 2 h at 30°C. Cells were fixed, permeabilized, and stained with antibodies to αIIbβ3 and c-Src and analyzed for BiFC and expression of αIIbβ3 and c-Src. The colored rectangle above each single black and white fluorescence image reflects the color of that protein in the merged image. (C) Cells as in B (i) were plated on fibrinogen and analyzed as indicated for BiFC, anti-αIIbβ3, and anti-cortactin (C, i) or BiFC, anti-vinculin, and rhodamine-phalloidin (C, ii). Colocalized regions for all three markers are white in the merged images. Histograms (mean ± SEM) indicate colocalization of BiFC with the indicated markers, and arrows in the zoomed image of the boxed area indicate colocalization at focal adhesions (white). This experiment is representative of five so performed.

instead of β3 (Fig. 4 A). Thus, αIIbβ3 ligation induces a proximal interaction between Syk and c-Src. Interactions between αIIbβ3, Syk, and c-Src were examined in more detail by BiFC using the chimeras shown in Fig. 2 A. Both Syk–c-Src and αIIbβ3–Syk BiFC complexes were detectable in fibrinogen-adherent cells but not in cells maintained in suspension. The results for the SykYC/SrcYN combination are illustrated in Fig. 4 (B and C). BiFC complexes involving Syk required an intact β3 cytoplasmic tail (Fig. 4 B), and they were present in membrane ruffles and lamellipodia, where they colocalized with cortactin, and in small focal complexes, where they colocalized with αIIbβ3 and vinculin (Fig. 4 C). However, BiFC complexes containing Syk were not detected in larger focal adhesions, which were identified by staining for vinculin and F-actin (Fig. 4 C, ii).

Real-time fluorescence microscopy revealed that Syk interactions with c-Src (Fig. 3 B and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200402064/DC1) or αIIbβ3 were dynamic, moving over an observation period of 6 min from membrane ruffles to small structures close to the cell periphery that resembled focal complexes. Thus, in contrast to αIIbβ3–c-Src complexes, which are constitutive and present at sites ranging from ruffles to mature focal adhesions, complexes involving Syk are induced by cell adhesion and confined primarily to ruffles and nascent adhesion structures close to the cell periphery. Like c-Src, Syk can bind directly to the β3 cytoplasmic tail in vitro (Woodside et al., 2001; Arias-Salgado et al., 2003), providing a potential structural explanation for these results. The reporter groups used here for BRET and BiFC were fused to the αIIb tail, not the β3 tail. However, because there are likely to be direct interactions between the αIIb and β3 tails (Vinogradova et al., 2002; Kim et al., 2003), the reporter on αIIb is presumably in sufficient proximity to the complementary reporter on c-Src or Syk (bound to β3) to enable these interactions to be detected in cells by BiFC or BRET.

These results raise additional questions. First, previous work with fibrinogen-adherent platelets and αIIbβ3-CHO cells has shown that Src activity promotes Syk activation (Gao et al., 1997; Obergfell et al., 2002). To determine if Src

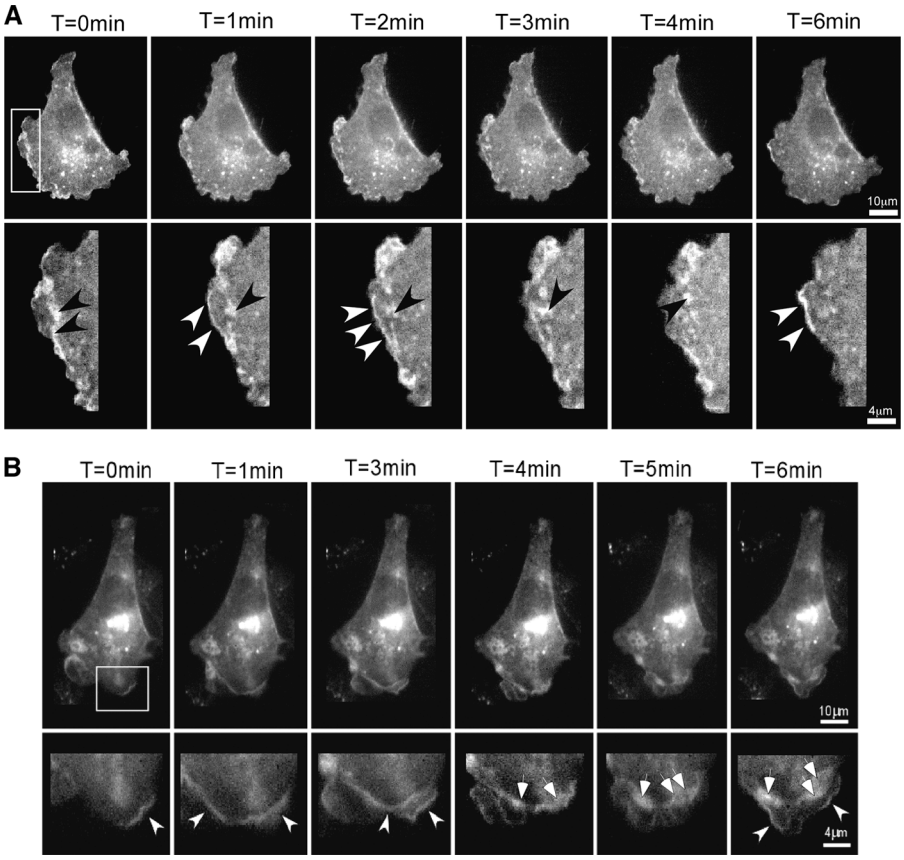

Figure 3. **Subcellular localization of αIIbβ3–c-Src and c-Src–Syk complexes detected by real-time BiFC.** Cells were transfected with αIIbYCβ3 and SrcYN (A) or SrcYC and SykYN (B). After 48 h, cells were plated on fibrinogen for 2 h at 30°C, and BiFC fluorescence was assessed for 6 min, as indicated. The bottom row of each panel zooms in on the boxed area shown in the top left panel. The white and black arrowheads indicate BiFC complexes at the tips of lamellipodia and in focal complexes and focal adhesions, respectively. In B, note BiFC complexes of Syk and c-Src primarily at the cell periphery in growing lamellipodia (arrowheads) and in focal complexes and focal adhesions (arrows). These results are representative of a total of 25 cells analyzed.

activity is required to recruit Syk to αIIbβ3, BiFC experiments were performed in the presence of a Src kinase inhibitor, either PP2 or SU6656 (Hanke et al., 1996; Blake et al., 2000). When 5 μM PP2 or 2 μM SU6656 was added to cells before plating on fibrinogen, interactions between αIIbYCβ3 and SykYN were partially inhibited, whereas the inactive congener PP3 had no effect (Fig. 5 A). Similarly, PP2 or SU6656 inhibited interactions between SykYC and SrcYN. When PP2 was added to cells already adherent to fibrinogen, it caused a rapid loss of BiFC signals between αIIbYCβ3 and SykYN or between SykYC and c-SrcYN (unpublished data). Thus, Src activity is required for the recruitment of Syk to αIIbβ3 and possibly for the maintenance of a critical density of integrin–Syk complexes in adherent cells.

Second, c-Src forms a complex with activated FAK in adherent cells, including platelets. In fibroblasts, this complex has been implicated in focal adhesion disassembly and cell motility, among other events (Parsons, 2003; Webb et al., 2003). To study proximal relationships involving FAK, FAKYN was coexpressed with SykYC, SrcYC, or αIIbYCβ3 (Fig. 2 A). In fibrinogen-adherent cells, BiFC was observed between FAKYN and αIIbYCβ3 and between FAKYN and SrcYC in focal complexes and focal adhesions (Fig. 5 B). However, FAK complexes were not detected in membrane ruffles, and interactions between FAKYN and SykYC were not detected anywhere. Thus, αIIbβ3 interactions with c-Src and Syk predominate in nascent adhesion structures, whereas those with c-Src and FAK predominate in focal adhesions. These relationships may help to explain previous biochemical and genetic data in platelets that implicate c-Src and Syk in initiating filopodia formation and actin polymerization, and FAK in later responses to adhesion (Lipfert et al., 1992; Clark and Brugge, 1993; Clark et al., 1994; Obergfell et al., 2002).

The current results serve to further emphasize the dynamic and heterogeneous nature of integrin-based signaling complexes (Miyamoto et al., 1995; Zamir and Geiger, 2001), and they reveal a new level of complexity in αIIbβ3 signaling. On a broader level, BRET and BiFC should provide a facile means to monitor binary interactions between integrins and many other proteins. Along with FRET and other recent elegant innovations in imaging (Kraynov et al., 2000; Webb et al., 2003), these two approaches should complement biochemical and genetic investigations into the molecular basis of integrin signaling.

Materials and methods

Reagents

Antibodies to Syk and cortactin were obtained from Santa Cruz Biotechnology, Inc. Antibodies 327 and Rb1671 were to c-Src (Arias-Salgado et al., 2003). Antibodies to αIIbβ3, Syk, and FAK were obtained from M. Ginsberg (The Scripps Research Institute, La Jolla, CA; Miranti et al., 1998; Obergfell et al., 2001). Cy5- and TRITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Rhodamine-phalloidin (Molecular Probes) was used to stain F-actin (Eugene); purified human fibrinogen was obtained from Enzyme Research Laboratories, Inc.; coelenterazine (Deep Blue C) was obtained from PerkinElmer; Src inhibitors PP2 and SU6656 were obtained from Calbiochem; and other reagents were obtained from Sigma-Aldrich.

Construction of recombinant proteins

For BRET assays, plasmid templates encoding human αIIb, Syk, or mouse c-Src were amplified by PCR to place appropriate restriction sites, and PCR products were subcloned into pGFPN2 and pRlucN2 mammalian expres-

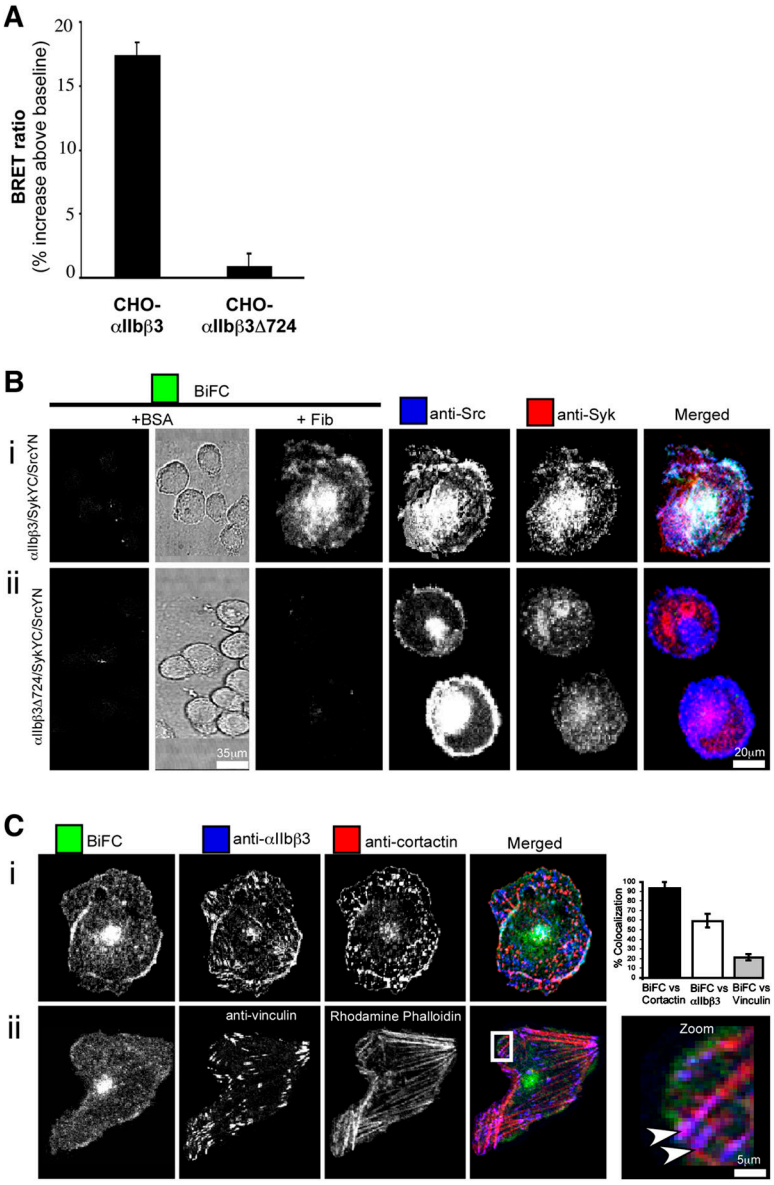

Figure 4. **Integrin-dependent interactions between Syk and c-Src detected by BRET or BiFC.** (A) Cells were transfected with the indicated cDNAs. 48 h later, BRET was analyzed in fibrinogen-adherent cells. The depicted baseline BRET ratio was that of suspension cells. Data are the means ± SEM of three experiments. (B) Cells were transfected with the indicated cDNAs, and BiFC was analyzed 48 h later in suspension (BSA) or fibrinogen-adherent cells (Fib) after fixation and staining with antibodies to c-Src and Syk. (C) Additional results for fibrinogen-adherent cells obtained under the same conditions as in B (i). Results are representative of five experiments. Histograms indicate colocalization of BiFC with the indicated markers. The high degree of colocalization of BiFC with cortactin and αIIbβ3 (white in merged images) is due to the abundance of these proteins and c-Src–Syk complexes in membrane ruffles. The relatively low colocalization of BiFC with vinculin is due to the absence of c-Src–Syk complexes from vinculin-rich focal adhesions. This is also observed by the absence of white regions in the merged image (C, ii) and in the zoom of the boxed area (arrowheads indicate focal adhesions).

sion vectors (Buensuceso et al., 2003) to produce the BRET chimeras in Fig. 1 A. For BiFC assays, plasmid templates encoding human αIIb, Syk, mouse c-Src, or the NH_2-terminal (YN) or COOH-terminal (YC) halves of YFP (a gift from T. Kerppola and C.-D. Hu, University of Michigan, Ann Arbor, MI; Hu et al., 2002) were amplified to place appropriate restriction sites, and PCR products were subcloned into pCDNA3 to produce the BiFC chimeras in Fig. 2 A. All coding sequences were verified by DNA sequencing, and expression plasmids were purified with the Qiafilter plasmid maxi kit (QIAGEN).

Cell culture and transfections

CHO cells were transiently transfected (Obergfell et al., 2001) with β3 and the appropriate BRET or BiFC chimeras. In experiments where interactions between two tyrosine kinases were being studied, the tyrosine kinase chimeras were transfected into A5 CHO cells stably expressing αIIbβ3 or αIIbβ3Δ724 (Obergfell et al., 2001).

BRET assays

48 h after transfection of BRET chimeras, CHO cells were resuspended to 3×10^6/ml in BRET buffer (Buensuceso et al., 2003), and 30 μl aliquots were added to microtiter wells precoated with 250 μg/μl fibrinogen or 5 mg/ml BSA . After 45 min at RT, 50 μl of luciferase substrate (coelenterazine, 10 μM final concentration) were added and BRET was determined by luminometry using a 410 nm/80 nm bandpass filter for Rluc and a 515 nm/30 nm bandpass filter for GFP. Results are expressed as the BRET ratio calculated as follows: (Emission at 515 nm − BG_{515})/(Emission at 410 nm − BG_{410}), where BG_{515} is the emission at 515 nm and BG_{410} is the emission at 410 nm of a 5-μM solution of coelenterazine prepared in BRET buffer (Xu et al., 1999; Buensuceso et al., 2003).

BiFC assays

48 h after transfection of BiFC chimeras, CHO cells were resuspended in Tyrode's buffer supplemented with 2 mM $MgCl_2$ and $CaCl_2$ and plated for 2 h at 30°C on coverslips precoated with 100 μg/ml fibrinogen to allow cell spreading and maturation of the YFP fluorophore (Hu et al., 2002). In parallel, cells were plated on 5 mg/ml BSA and maintained in suspension. Cells were fixed in 3% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with primary and secondary antibodies. Fluorescence images were acquired with a laser scanning confocal microscope (model MRC 1024; Bio-Rad Laboratories) and processed with imaging software (ISee Imaging Systems). Colocalization of BiFC fluorescence with other fluorescence images was quantified at the single pixel level, and data were accumulated for at least 20 cells over three experiments. In accordance with current definitions, a focal complex was defined here as a round, integrin-based structure smaller than 1 μm^2, located 0–3 μm from the leading edge of a migrating cell, whereas a focal adhesion was defined as an elongated, integrin-based structure of 2–3 μm^2 linked to actin stress fibers that could be located throughout the basal surface of the cell (Burridge et al., 1988; Jockusch et al., 1995). To monitor BiFC in real time (Kiosses et al., 1999), cells were plated on fibrinogen for 90 min at 30°C. Images were

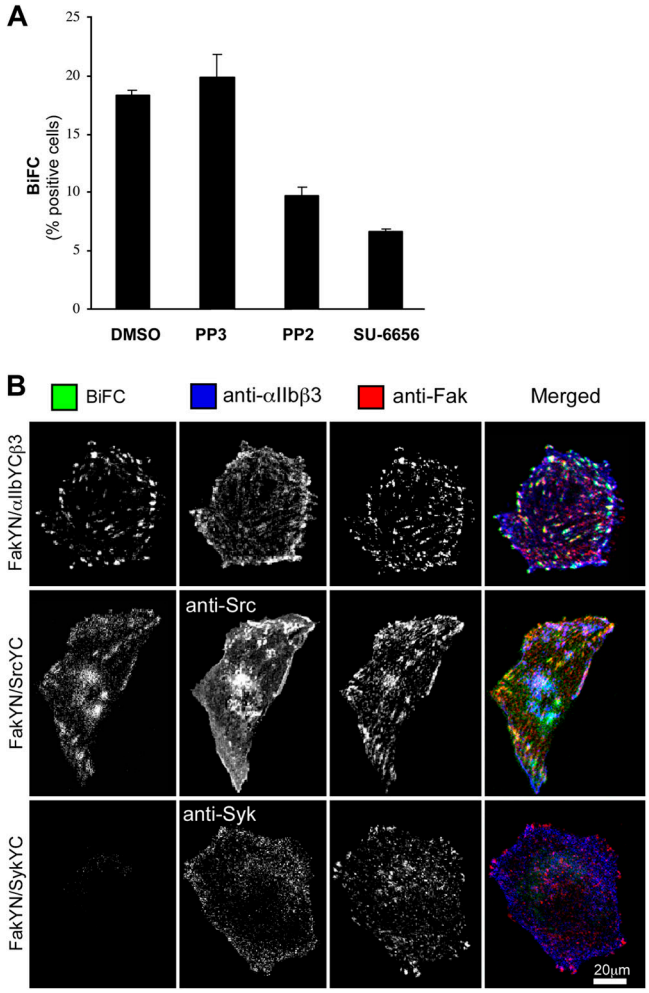

Figure 5. **Relationships between Syk, c-Src, and FAK in fibrinogen-adherent cells.** (A) Src activity promotes formation of αIIbβ3–Syk complexes. CHO cells were transfected with cDNAs for αIIbYCβ3 and SykYN. 48 h later, they were incubated in suspension for 30 min at RT with DMSO vehicle, 5 μM PP2, 5 μM PP3, or 2 μM SU6656. After plating on fibrinogen for 2 h at 30°, the percentage of BiFC-positive cells was quantified. At least 100 cells were analyzed per sample. Data are means ± SEM of three experiments. (B) Cells were transfected with cDNAs as indicated, and after plating on fibrinogen, the localization of BiFC and specific proteins was assessed. Note the presence of BiFC complexes of αIIbβ3 and FAK (top) or c-Src and FAK (middle) in focal adhesions. In contrast, BiFC complexes of Syk and FAK were not detected (bottom). Results are representative of three experiments.

captured using an inverted fluorescence microscope (model TE300; Nikon) equipped with a CCD camera (Model Micromax 1024B ; Roper Scientific) and analyzed with ISEE imaging software.

Online supplemental material

Video 1 shows interactions between αIIbβ3 and c-Src. This Quicktime movie shows a BiFC signal in real time between αIIbYCβ3 and c-SrcYN in a CHO cell starting 90 min after plating on fibrinogen. Fluorescence images were acquired at 1-min intervals (1 frame/min) for 34 min as described in Materials and methods. The movie is repeated a second time zooming in on a region of growing lamellipodium and shows the BIFC signal at the cell periphery moving inward to focal complexes and adhesions.

Video 2 shows interactions between c-Src and Syk. This Quicktime movie shows a BiFC signal in real time between c-SrcYN and SykYC in a CHO cell starting 90 min after plating on fibrinogen. Fluorescence images were acquired at 1-min intervals (1 frame/min) for 54 min. The movie is repeated three times over a 9-min period zooming in on a region of growing lamellipodium that shows the BiFC signal at the cell periphery moving inward and reappearing always first at the cell edge. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200402064/DC1.

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