Dynamic Interaction between Src and C-terminal Src Kinase in Integrin αIibβ3-mediated Signaling to the Cytoskeleton

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Integrin-bound Src tyrosine kinase mediates αIibβ3 outside-in signaling to the cytoskeleton required for platelet adhesion and thrombus formation. Src activation (signal initiation) by phosphorylation of Tyr-418 occurs at lamellipodia leading edges. However, little is known about Src inactivation mediated by C-terminal Src kinase (Csk) Tyr-529 phosphorylation. In an established platelet model cell line (A5-Chinese hamster ovary), we studied the inactivation of Src during αIibβ3-mediated adhesion to fibrinogen with live cell fluorescence resonance energy transfer (FRET) microscopy. Imaging revealed highly dynamic Src-Csk interactions at the leading edges of active lamellipodia. The Src-Csk interaction followed a highly dynamic pattern. Every 2–3 min, Src-Csk complexes moved inward in the cell, reorganized, and formed stable focal adhesions. These accumulations were primarily seen during retraction of lamellipodia, whereas no interaction was observed during protrusions. Western blot analysis during the run time of FRET signaling revealed an increase in Csk-mediated SrcTyr-529 phosphorylation with a parallel decline of tyrosine 418 phosphorylation. Mutation analysis provided additional insights into the role of Src. Although inactivation of Csk (CskK222R) had no effect on cell adhesion and spreading efficiency, cells with constitutively active expressed Src (SrcY529F) exhibited hardly any adhesion and no spreading. The few adherent cells showed weak focal adhesions that were disorganized and oversized. The data clearly demonstrate the important role of tight Src control by Csk for functional cell adhesion and spreading. The novel experimental FRET approach reported here for the inactivation of Src can be readily applied to other integrin and signaling pathways, including closely related Src family kinase members.

Integrins are heterodimeric α/β-transmembrane adhesion receptors that bind with their extracellular domains to proteins of the extracellular matrix or to counter-receptors on other cells and signal with their cytoplasmic part to the actin cytoskeleton (1–3). The signaling pathway from integrins to the actin cytoskeleton (outside-in signaling) is mediated by a network of tyrosine kinases (e.g. Src and Syk), phosphatases, and adaptor proteins. In platelets, fibrinogen binding to the αIibβ3 receptor activates Src tyrosine kinases initiating downstream signaling (4). Src thereby regulates the interaction between integrin and the cytoskeleton (5).

From extensive studies with platelets and platelet model cells (A5-CHO), the role of Src in αIibβ3 adhesion signaling is well described (6). Membrane-anchored Src is constitutively bound to the β3 cytoplasmic tail of the fibrinogen receptor. Fibrinogen binding at lamellipodia leading edges causes microclustering of integrins thus leading to trans-autophosphorylation at Tyr-418, conformational opening, and full activation of Src.

Many proteins are known to influence Src activation in platelets. However, inactivation is controlled only by Csk (7, 8). With its Src homology 2 domain, Csk binds to the C-terminal tail of active Src thereby phosphorylating Tyr-529. This chemical clamp mechanism controls Src recognition (9). The intramolecular interaction of Tyr(P)-529 with the Src homology 2 domain leads to the conversion of Src back to the inactive (closed) conformation with Csk bound to the integrin-Src complex (4). This Csk-mediated inactivation is observed with all members of the SFK (8).

Src is ubiquitously expressed in mammalian cells and is, in addition to platelet integrin signaling, involved in other distinct pathways and cellular targets (G-protein-coupled receptors, cytokine and immune recognition receptors, and ion channels) (7). Src is the first discovered and best studied proto-oncogene. Its role in focal adhesions is associated with cell detachment, migration, and invasion that is important in the spreading of cancer (10).

Increased Src activity has been demonstrated in a number of human malignancies, and control of Src by Csk was associated with the progression of cancer (10). Higher Src activity or low levels of Csk weakened focal adhesions and supported cell detachment.

In nonmigrating cells, Csk was found to co-localize with Src in stable focal adhesions (11). Csk overexpression was also
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found to strengthen focal contacts as seen in a mouse model where overexpression of Csk suppresses metastasis (11). These results support that Src inactivation mediated by Csk plays an important role in the regulation of focal adhesion formation and stability. However, location and dynamics of this interaction and its role in αIIbβ3-mediated adhesion remain unclear.

To address the question of Src inactivation, we used FRET, a noninvasive and stable technique for the imaging of live cell protein–protein interactions that is superior over other protein interaction reporter assays (12–14). FRET is a physical effect (energy transfer) between fluorescent donor (e.g. cyan fluorescent protein (CFP)) and acceptor molecules (e.g. yellow fluorescent protein (YFP)) that only occurs efficiently within the range of molecular interactions.

A5-CHO cells, stably expressing human integrin αIIbβ3, were transiently transfected with Src and Csk and labeled with FRET acceptor (YFP) and donor (CFP) molecules, respectively. The Src and Csk interaction was then visualized and tracked in single cells during adhesion on a fibrinogen-coated glass coverslip.

This study reveals insights into the inactivation of integrin αIIbβ3 signaling and contributes to a better understanding of adhesion signaling in platelets. The presented FRET approach is currently the only system that reports on Src inactivation and may be used in other cells and pathways. Its extension and use in SFK-mediated integrin signaling pathways may also help for a better understanding of some behavioral aspects of cancer cells.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Total Src was detected with mouse monoclonal antibody 327 (Sigma) and active and inactive Src with phosho-specific rabbit polyclonal anti-Src-Tyr(418) and anti-Src-Tyr(529) (BIOSOURCE). Antibodies against Csk (antibody 52) and integrin β3 (antibody SAP) were from BD Biosciences and Santa Cruz Biotechnology, respectively. HRP-conjugated secondary antibodies were from Bio-Rad.

Purified human fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN). L-Glutamine, nonessential amino acids, and Alfazyme (mild detachment agent) were from BD Biosciences and Santa Cruz Biotechnology (Heidelberg, Germany), respectively. HRP-conjugated secondary antibodies were from Bio-Rad.

DNA Constructs—Constructs coding for Csk (rat) were kind gifts from S. Nada (Dept. of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Japan) (15). Csk_wt (wild-type) and the FRET-labeled Csk_wt-CFP and CskK222R-CFP (kinase-inactive) (Fig. 1) were used. For optimal FRET results, CFP in Csk_wt-CFP was replaced by the optimized CFP variant Cerulean (obtained from C. Krasel, Dept. of Pharmacology, University of Wurzburg, Germany) (16) using a PCR-based strategy.

Constructs coding for chicken Src were a kind gift from S. J. Shattil (Dept. of Medicine, Hematology-Oncology Division, University of California, San Diego, La Jolla). In this study we used Src_wt, SrcK295R (kinase-inactive), and SrcY527F (constitutively active) pLNCX constructs (17, 18). For simplicity reasons, Tyr-527 is named Tyr-529 here following the human numbering.

All Src constructs were labeled with an improved YFP variant, Venus (19). Venus, cloned into the XbaI/XhoI sites of pcDNA3, was received from J.-P. Vilardaga (Dept. of Pharmacology, University Wurzburg, Germany). Src coding sequences were amplified by PCR using primers that introduce BamHI and EcoRI sites and then cloned upstream of Venus in pcDNA3-Venus with the linker EFCRYPHPHRPLET (Fig. 1) and are referred to as Src-YFP constructs.

Cell Culture and Transfections—A5-CHO, stably overexpressing human integrin αIIbβ3 (20, 21), was a gift from M. Ginsberg (Dept. of Medicine, University of California, San Diego). Cells were cultured in growth medium (D5671) as described (22) for a maximum of 2 weeks ahead of use to maintain constant integrin expression.

Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations at 90% cell density. To avoid cell density stress, cells were split 1:3 after 24 h and cultured for another 24 h.

Cell Lysis and Immunoblotting—Cells were harvested (Alfazyme), washed twice with PBS, and lysed for 10 min on ice with RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing phosphatase inhibitor mixture 2 (Sigma), 10 mM NaF, and protease inhibitors (Complete, Roche Diagnostics). After centrifugation (2 min, 16,000 × g) protein concentrations were determined with the biuret reaction (BCA assay, Pierce).

Five μg of total protein were separated on 7.5% SDS-polyacrylamide gels and transferred by Western blot on nitrocellulose membranes (Hybond-P, Amersham Biosciences). Proteins were detected using the indicated primary and horseradish peroxidase (HRP)-conjugated secondary antibodies with enhanced chemiluminescence (ECL Plus, Amersham Biosciences).

Co-immunoprecipitation—Cells were harvested in RIPA/lysis buffer without deoxycholic acid (inhibitors as described). After clarification, 500 μg of cell lysate was incubated with 2 μg of an antibody specific to β3 integrin subunit (N-20, Santa Cruz Biotechnology) for 2 h at 4°C followed by another 2-h incubation with protein G-Sepharose beads (protein G-Sepharose 4 fast flow; Amersham Biosciences). The beads were washed three times with lysis buffer. Protein complexes were released by 5 min of boiling in SDS-PAGE sample buffer, separated by electrophoresis, and immunoblotted on nitrocellulose. Proteins were detected using the indicated primary antibodies and HRP-conjugated secondary antibodies as described.

Src Phosphorylation Assay—A5-CHO cells in 100-mm plates were co-transfected with Src_wt and Csk_wt (or Src_wt-YFP and Csk_wt-CFP) constructs and cultured for 2 days (as described above). Alfazyme was used for harvesting (instead of trypsin) to prevent preeactivation artifacts that may derive from partial integrin αIIbβ3 digestion. Cells were diluted in 6 ml of complete culture medium (without penicillin/streptomycin) and incubated for 1 h at 37°C, 6% CO2 (resuspension by mild agitation every 10 min).

Then cells were plated on 100-mm dishes pre-coated with fibrinogen (100 μg/ml) or BSA (5 mg/ml) according to Ref. 23

4 S. J. Shattil, personal communication.
and incubated for the times indicated at 37 °C, 6% CO₂. Fibrinogen-adherent cells were washed twice with ice-cold PBS, overlaid with 600 μl of ice-cold, complete lysis buffer, scraped off, and lysed completely for 10 min on ice. Nonadherent cells from BSA plates were washed twice with ice-cold PBS and lysed for 10 min on ice.

After centrifugation and testing with BCA assay methods, equal amounts of total protein were blotted and immunodetected for total, active, and inactive Src. As controls, platelet lysates were used (0.05 units/ml thrombin-stimulated platelets for activated Src; unstimulated platelets for inactive Src). Phosphorylated Tyr-418 (Tyr(P)-418) is the marker for active Src and Tyr(P)-529 for inactive Src.

Bands on the x-ray film were scanned and quantified by calibrated densitometry using ImageJ software (National Institutes of Health, Bethesda). Amounts of active and inactive Src were expressed in a line graph as normalized percentages. Therefore, the levels of Src_Tyr(P)-418 and Src_Tyr(P)-529 were expressed relative (in %) to the highest respective level present (100%).

Cell Preparation for Microscopy—Cells transfected with Src-YFP and Csk-CFP constructs (60-mm dish) were harvested with Alfazyme 2 days post-transfection, resuspended in 2 ml of phenol red-free and serum-reduced medium (D1145, 1% fetal bovine serum, other supplements as in growth medium, no penicillin/streptomycin), and incubated as described for 1 h at 37 °C.

Cells were plated on fibrinogen-coated number 1 glass coverslips (Hartenstein, Würzburg, Germany), clamped in a coverslip chamber, and incubated for 25 min at 37 °C, 6% CO₂. Nonadherent cells were removed by washing twice with warm PBS, and adherent cells were overlaid with warm incubation medium and immediately transferred to the microscope. Single cells with intermediate CFP and YFP fluorescence (~1:1) were selected and focused to lamellipodia regions.

Confocal Fluorescence and FRET Localization—Measurements were performed at room temperature on a confocal microscope (TCS-SP2, Leica, Mannheim, Germany). This microscope was equipped with a ×63 objective (NA 1.32, oil), two laser lines (430 nm for CFP or FRET and 514 nm for YFP excitation), and excitation beam splitters RSP455 (430 nm) and DD458/514 (514 nm). The fluorescence was detected by 2 high efficiency photomultipliers (CFP, 463–496 nm; YFP and FRET, 549–600 nm). Cells were scanned at 400 lines/s and with four times averaging. Images (512 × 512 pixels) were optimized and overlay images (YFP/CFP) created with ImageJ.

FRET was calculated according to the sensitized emission methods of Ref. 24 in which the FRET, CFP, and YFP emission channels were recorded sequentially line-by-line per recorded image. Intensities in FRET images were color-coded (ImageJ). From time series images (every 25 s) videos (“.avi” format) were generated in ImageJ. For more details of the sensitized emission FRET calibration and calculations, see the Supplemental Material.

Fluorescence Detection and FRET Efficiency Imaging with Wide Field Microscopy—The microscopic setup used was described previously (25). A ×63 objective (N.A. 1.4, oil) was used. Donor excitation occurred at 430 nm, acceptor excitation at 514 nm, and both wavelengths of equal intensity (at the objective, ~10 watts/cm²) were successively switched on (time lag, 100 ms) for 100–400 ms (depending on relative CFP and YFP expression in the individual cells) to obtain fluorescence signals of comparable intensity. Frames were taken every 5 s to track fluorescence or FRET changes.

The method has been previously described (25). FRET efficiency (EFRET) images (150 × 150 pixels) were calculated in ImageJ according to Equation 1,

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EFRET = \frac{I_{YFP}}{I_{YFP} + I_{CFP}}
\]

(Eq. 1)

I_{YFP} and I_{CFP} are the intensities in the YFP and CFP channel at 430 nm excitation corrected for spectral bleed through factors.

Acceptor photobleaching measurements were performed in lamellipodia and proved the presence of FRET by a significant increase in CFP and decrease of the FRET signal after total acceptor (YFP) bleaching.

FRET images were color-coded (ImageJ) to better discriminate between signal intensities. Videos (in .avi format) were produced in ImageJ. For more detail on FRET efficiency (EFRET) calibration and calculations, see the Supplemental Material.

Immunofluorescence Co-staining of Vinculin—For vinculin immunostaining, Src_wt-YFP/Csk_wt-CFP transfected A5-CHO were plated on fibrinogen-coated glass chamber slides for 20, 30, and 45 min, fixed in 4% (w/v) para-formaldehyde in PBS, permeabilized with 0.1% (w/v) Triton X-100 in PBS, and then stained with monoclonal vinculin antibody (1:50, Sigma) followed by secondary Cy3-labeled anti-mouse antibody (Dianova, Hamburg, Germany).

The co-localization of the Cy3-vinculin, Csk-CFP, and Src-YFP was imaged with a confocal microscope (TCS-SP5, Leica, Mannheim, Germany). This microscope was equipped with a ×63 objective (N.A. 1.4, oil), three laser lines (405 nm for CFP or FRET, 514 nm for YFP, and 561 nm for Cy3 excitation), and an acousto-optic excitation beam splitter for all laser excitation lines simultaneously. The fluorescence was detected by three high efficiency photomultipliers (CFP, 472–502 nm; YFP and FRET, 520–556 nm; and Cy3, 582–692 nm). The fixed cells were scanned at 400 lines/s and with eight times averaging.

Calculation of Protein Expression Levels in FRET Cells—The emission rate of single YFP and CFP molecules as a function of laser excitation intensity was determined on the wide field microscopy setup described above (26, 27). With the applied excitation times, laser intensity, mean emission wavelengths, and objective conditions (×63, N.A. 1.4, oil, depth of focus 0.041 μm) the amount of detected YFP and CFP molecules recorded on the CCD camera were approximated. FRET effects in these calculations were negligible (supplemental Fig. 1).

RESULTS

Src-YFP and Csk-CFP Expression—For the detection of Src-Csk interactions in living cells with FRET microscopy, the proteins need to be labeled with CFP and YFP. Csk was labeled N-terminally with CFP because this construct had already been successfully used in another FRET study (25). Src-YFP constructs were generated by introducing a 14-amino acid linker to
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the Src C terminus to facilitate Tyr-529 phosphorylation by Csk (Fig. 1). The expression of labeled and unlabeled Src and Csk constructs was tested by Western blotting after single transfection of A5-CHO cells (Fig. 2). Src wt and SrcY529F were detected at 60 kDa and Src wt-YFP, SrcY529F-YFP, and SrcK295R-YFP at 87 kDa (YFP, 27 kDa). Csk wt and CskK222R as well as Csk wt-CFP and CskK222R-CFP were detected at 43 and 70 kDa, respectively (CFP, 27 kDa) (Fig. 2B). The endogenous expression of Src and Csk was negligible for later FRET signaling as determined by Western blot analysis.

In cells co-transfected with Src wt and Csk wt constructs, clear expression of both proteins was observed (Fig. 2C) (similar results for labeledSrc and Csk; data not shown). Expression of integrin $\alpha_{IIb}\beta_3$, stably overexpressed in A5-CHO, was verified with an antibody specific to the $\beta_3$ chain. The integrin was found to be highly expressed, and the expression level was not altered by transient expression of Src and Csk (Fig. 2, B and C).

From single molecule studies examining the fluorescence emission of the YFP and CFP variants, the expression levels of Src and Csk in the monitored cells was calculated to be 2 $\mu$M for Src wt-YFP and 4 $\mu$M for Csk wt-CFP. In platelets Src concentrations have been determined to be $\sim 25$ $\mu$M (supplemental Fig. 1).

Csk-CFP/Src-YFP Co-immunoprecipitation—For Src-mediated integrin outside-in signaling in platelets, the interaction of Src with $\alpha_{IIb}\beta_3$ is critical. Therefore, we performed co-immunoprecipitation experiments to demonstrate the interaction of Src with the integrin. As seen in Fig. 2, D and E, in lysates from fibrinogen-adherent A5-CHO cells Src wt was detected in $\beta_3$ immunoprecipitates in complex with Csk wt. The association was independent of FRET labeling. Interestingly, the Csk-kinase-dead mutant CskK222R is still binding to Src wt, whereas no binding is observed between permanent active SrcY529F and Csk wt (Fig. 2E).

Csk-CFP/Src-YFP Interaction during Adhesion to Fibrinogen—Because FRET signaling between labeled Src and Csk (showing Src-Csk interactions) has only limited value without biochemical proof of real Src inactivation, we tested Src inactivation in A5-CHO cells co-expressing Src wt-YFP and Csk wt-CFP during the course of adhesion to fibrinogen. After plating for 20, 30, and 45 min on BSA- or fibrinogen-coated plates, cell lysates were tested for Src_Tyr(P)-529 (inactive), Src_Tyr(P)-418 (active), and total Src content (Fig. 3A).

Whereas the phosphorylation status of Src was not altered after plating up to 45 min on a BSA-coated surface (Fig. 3A, BSA), specific changes in the amount of Src_Tyr(P)-529 and Src_Tyr(P)-418 phosphorylation were observed after spreading on fibrinogen. After 20 min of plating, the relative amounts of inactive Src clearly increased whereas active Src in total decreased (Fig. 3A, Fibrinogen). Relative percentages of band intensities from densitometric analysis illustrate this trend (Fig. 3B). Identical results were obtained from cells co-expressing unlabeled proteins (data...
not shown). In A5-CHO cells transfected with either Src_wt alone or Src_wt in combination with CskK222R, no change in the phosphorylation status of Src was observed (Fig. 3).

Before plating, cells already exhibited a considerable amount of pre-phosphorylated Src. Control experiments with cells transfected with Src alone also showed this high pre-activation (data not shown) assuming that the amount of Tyr-418 phosphorylation seen in the Western blots of Fig. 3A is, at least in part, because of incomplete single-transfected cells expressing only Src without the inhibitory modulator Csk. However, for the FRET experiments only double transfected cells were used.

**Src Co-localization by Csk Occurs at the Leading Edges of Lamellipodia**—To assess the subcellular localization of Src_wt-YFP and Csk_wt-CFP, confocal fluorescence images were taken from the basal surface of A5-CHO cells 30 min after plating on fibrinogen. Fig. 4 depicts cells with typical morphology and protein localization.

Src_wt-YFP is targeted to the plasma membrane and partially accumulated at the edges of some lamellipodia (Fig. 4, white arrows) and in focal adhesions (green arrows). Cytoplasmic Csk_wt-CFP was also partially accumulated to lamellipodia edges (Fig. 4B, white arrows). A detailed view into lamellipodia is given in the overlay image (Fig. 4C, Src_wt-YFP in green and Csk_wt-CFP in red). Both proteins are partially co-localized (Fig. 4C, yellow) both at the leading edge (white arrow) and in some focal adhesions (green arrows).

Interactions between Src_wt-YFP and Csk_wt-CFP 25 min after adhesion were tested with wide field FRET microscopy (Fig. 4, D–F). Interactions were calculated and expressed as FRET efficiencies (EFRET, in %) (Fig. 4, D–F). Positive EFRET was only seen in cells with low to medium expression and about equal amounts of both Src_wt-YFP and Csk_wt-CFP (EFRET > 25%). These signals were specific to fibrinogen stimulation, because in BSA-plated cells the EFRET levels reached only about 15% (Fig. 4F).

Positive FRET signals (>25%) were primarily located at the leading edges of lamellipodia (Fig. 4D, red arrows), although inside the cell the FRET intensities were much lower (18–25%). This distribution was only seen in cells with active lamellipodia, whereas in completely adherent and spread cells a uniform distribution (at a low level) was observed (not shown) supporting a correlation between Src-Csk interaction and lamellipodial spreading. To study the dynamic changes in FRET, we recorded time-lapse images in 5-s intervals. Two observations were made (Fig. 4E).

First, FRET intensities increased quickly (Fig. 4E, upper lamellipodium, black arrows at 0 and 25 s or tip of white boxed lamellipodium at 0 and 15 s). Second, areas of positive FRET moved inward in the cell very dynamically (white-boxed lamellipodium, white arrowheads at 0, 10, 20, and 30 s). The movement can easily be tracked in images revealing Src_wt-YFP localization (black and white images shown below FRET sequence).

We determined a fast velocity of ~90 nm/s (supplemental Video 2). The real time process can be seen in supplemental Video 1.

**Detailed Analysis of FRET Dynamics**—To increase resolution and to localize FRET signals in more detail, thinner sectioned confocal microscopy was used. In Fig. 5A, Src-Csk interactions in lamellipodia (red arrows) and in focal adhesions (green arrows) are shown in high resolution.

The dynamic behavior of these signals was tracked over 125 s in 25-s intervals (white-boxed images). The dynamic events can be described as follows: FRET signals first accumulate along the leading edge (Fig. 5A, red arrows, here shown at 0 s). After 1–2 min the signal begins to move inward in a characteristic manner (Fig. 5A, white arrows, 25 and 50 s). During the inward motion, the leading edge protrudes outwardly or in the opposing direction (Fig. 5A, blue arrows at 25 and 50 s).

Inward moving signals become fragmented (Fig. 5A, white arrows, 75 s) and finally stably locate to focal adhesions further inside (green arrows, 100 s) as shown by co-immunostaining...
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Expression of Src_wt-YFP / Csk_wt-CFP

A  Src_wt-YFP  B  Csk_wt-CFP  C  Overlay

FIGURE 4. Localization and FRET of Src_wt-YFP and Csk_wt-CFP during fibrinogen adhesion. Cells expressing Src_wt-YFP and Csk_wt-CFP on fibrinogen-coated coverslips were imaged after 25 min of adhesion. A–C, protein localization imaged with confocal fluorescence microscopy (×63). A, membrane-targeted Src_wt-YFP locates to focal adhesions (green arrows) and accumulates at the edges of lamellipodia (white arrows, together with cytoplasmic Csk_wt-CFP (in B, white arrows)). C, overlay image zooms into the boxed region in B (Src_wt-YFP in green, Csk_wt-CFP in red) and shows in more detail the Src and Csk localizations. Partial colocalization (yellow) is visible in lamellipodia leading edges (white arrow) and in focal adhesions (green arrows). D–F, Src-Csk interaction imaged with wide field FRET microscopy (×63). FRET efficiencies range from −15 to 30% (color bar). D shows FRET in a single cell whereas E shows 2 lamellipodia of the same cell in an image sequence with the corresponding Src_wt-YFP localization sequence for comparison. Protein interaction mostly occurs in lamellipodia and their leading edges (D, red arrows, EFRET > 25%) and dynamically relocates in moving lamellipodia (E). An inward moving FRET signal wave appears in the white-boxed lamellipodium in E (white arrows). Control cells plated to BSA-coated coverslips exhibit only low FRET levels (F, 15–20%). Cells are representative for at least 20 typical cells imaged.

FIGURE 5. Detailed analysis of the events of Src-Csk interaction in lamellipodia. In a series (every 25 s) of confocal FRET images (×63) both location and mobility of Src-Csk complexes become obvious. Two representative cells expressing Src_wt-YFP and Csk_wt-CFP (A and B) are shown. A visualizes locations of Src-Csk interactions with high resolution. After Src inactivation at the leading edge (red arrows, 0 s), FRET signals move inward (white arrows, 25 and 50 s), thereby fragmentize (75 s), and get located to focal adhesions (green arrows, 100 s). Beginning with start of inward motion lamellipodia protrusion occurs (blue arrows at 25 and 50 s). About 100 s after initial clustering, FRET reappears at the leading edge (red arrows at 100 and 125 s). B, here in this cell with a fast moving lamellipodium, the role of Src inactivation in lamellipodial growth becomes obvious. FRET signal clustering can be seen during retraction (red arrows directing inwards at 50, 200, and 300 s), whereas no clustering occurs during protrusion (blue arrows directing outward: 0, 150, 200, and 250 s). Src inactivation is likely to inhibit lamellipodial growth. Cells are representative for at least 15 individual cells imaged each.

with the focal adhesion marker vinculin (supplemental Fig. 3). After minutes (here after 100 s, red arrow) FRET signals again accumulate at the leading edge restarting the process (Fig. 5A, 100 and 125 s). Supplemental Videos 2 and 3 give an impression of this dynamic event in different cells.

FRET appearance at the leading edge could be correlated to lamellipodia movements in a cell with strong lamellipodia growth activity (Fig. 5B). The image sequence clearly shows that during phases of protrusion FRET is not present at the leading edge (Fig. 5B, blue arrows at 0, 150, and 250 s). In non-protruding or retracting areas, however, FRET strongly accumulates at the edge (Fig. 5B, red arrows at 50, 200, and 300 s). Src-Csk interaction at the leading edge of lamellipodia obviously stops and inhibits protrusion, as complex inward transport again triggers lamellipodial growth. This motion can be observed in supplemental Video 4.

Src Inactivation Is Required for Adhesion and Spreading—To gain further insight into the role of Src-Csk interactions for cell adhesion and spreading, we imaged cells expressing mutant Src-YFP or Csk-CFP proteins. In the first experiment, we used SrcY529F-YFP, a constitutively active Src mutant, which cannot be inactivated by Csk, and co-expressed it in A5-CHO cells with Csk_wt-CFP. These cells show strong proliferation rates in culture (not shown). After plating on fibrinogen, only a small number of cells attached but were not able to spread (Fig. 6, A–D). Attached cells were shaped like irregular formed columns (not shown).

Both proteins were located in focal adhesions, but the structures were strongly accumulated and oversized, leaving them distributed in a nonuniform fashion (Fig. 6, A and B, green arrows). Protein co-localization (yellow) was hardly seen (Fig. 6C, green arrows).

The cells also exhibited strong formation of membrane blebs. FRET image (Fig. 6D) shows that protein interaction is not present in the bleb membrane (white arrows).

In Supplemental Video 5, the bleb forming dynamics can be followed. Bleb formation occurred highly dynamically all around the cell (new blebs were seen within only 25 s). Blebs can...
Expression of SrcY529F-YFP / Csk_wt-CFP

A: SrcY529F-YFP  B: Csk_wt-CFP  C: Overlay

D: FRET  E: Expression of SrcY529F-YFP  F: Expression of Src_wt-YFP

Expression of Src_wt-YFP / CskK222R-CFP

G: Src_wt-YFP  H: CskK222R-CFP  I: Overlay  K: FRET

FIGURE 6. Src and Csk mutations reveal the importance of Src inactivation for cell adhesion. Cells were pretreated and measured with confocal microscopy (×63) as in Figs. 4 and 5. A–C and G–I, proteins were localized by fluorescence imaging; D and K, their interactions were imaged with FRET. A–D, in cells expressing constitutively active SrcY529F-YFP together with Csk_wt-CFP, cells hardly adhere. Proteins are found in large, disorganized spots (A–C, green arrows). FRET (D) is nearly absent in focal adhesions and in the membrane, and blebs are visible (white arrows). These blebs (white arrows) are also seen in single SrcY529F-YFP transfected cells (E), whereas cells with individual expressed Src_wt-YFP show a normal morphology (F). G–K, cells expressing Src_wt-YFP together with kinase-inactive CskK222R-YFP effectively adhere and spread, but they exhibit abnormalities in the lamellipodia like strong formation of filopodia (G and I, white arrowheads) and small rough growing regions on a single broad lamellipodium (H, white arrow, and K, white bracket/blue arrowheads). CskK222R-CFP is heavily mislocalized in lamellipodia regions (sharp border in H and I, red arrows). Clear FRET signals are visible between inactive Csk and wild-type Src. However, their cellular distributions are changed (K, red arrows). Cells are representative for at least 20 individual cells imaged each.

The cells showed a high efficiency of adhesion and spreading that was comparable with normal cells (Figs. 4 and 5). However, a number of striking structural and functional abnormalities was observed. First, CskK222R-CFP localization was strongly affected (Fig. 6H). Although normal localization was seen in the cell center, only minor levels were present in the lamellipodia (Fig. 6H, except for their edges, white arrow), and a sharp border at the interface between lamellipodia and lamella (28) separated these two regions (red arrow in Fig. 6, H and in G/I). Second, the cells appeared rough with a large number of filopodia showing Src localization (Fig. 6, G and I, white arrowheads). Finally, the spreading phenotype was altered characterized by formation of very large lamellipodia (Fig. 6K, white brackets) being not as structured as in normal cells (Fig. 4C).

Unexpectedly, clear Src-Csk interactions (FRET) were detected in the cells (Fig. 6K). However, the distribution of FRET complexes was clearly different compared with cells expressing the wild-type proteins (Fig. 5A). Interactions accumulate along the periphery of lamellipodia (Fig. 5A, red arrows). Small, local areas of protrusion (Fig. 5A, blue arrows) are seen in these flat and very broad lamellipodia (FRET is not present in these protrusions).

In supplemental Video 6, it becomes evident that the FRET complexes are only slightly mobile and remain localized, meaning that no fragmentation to focal adhesions occurred. The lamellipodia are also only barely mobile, and constantly and slowly protrude with no retractions. Our experiments with inactive Csk show that Src inactivation is clearly required for lamellipodia and focal adhesion function.

DISCUSSION

Integrin outside-in signaling affects a number of cellular responses, including reorganization of the actin cytoskeleton important for the regulation of lamellipodial activity and focal adhesion function. The Src tyrosine kinase protein regulates the integrin-cytoskeleton interaction and acts as a switch that is tightly and negatively controlled by Csk (5, 8). Much information concerning the role of Src in platelet integrin α_{IIb}β_{3} outside-in signaling (6) was gained in the last years (4, 29, 30). We were able to create a FRET labeling system that visualizes not only the Src-Csk interaction during fibrinogen adhesion and
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\( \alpha_{\text{IIb}}\beta_3 \) signaling but also reports on the role of Src for lamellipodia function and focal adhesion.

The following results were obtained by FRET imaging. (a) Src inactivation occurs at the leading edges of nascent lamellipodia. (b) Complexes of integrin-Src-Csk are periodically removed from the leading edge and translocate to focal adhesions. (c) Src-Csk complex formation ceases, and complex removal restarts the protrusion of lamellipodia.

Several interaction reporter assays have been applied to visualize the events of Src activation. So far, these assays have only been able to show locations of Src activity (31) or interactions closely related to Src activation (15, 30) but not the processes of Src inactivation. Wang et al. (31) created a single molecule sensor of Src activity that responded to Src phosphorylation with intramolecular FRET changes. In another study, events directly following Src activation (Csk activation by interaction with Cbp) in epidermal growth factor signaling were shown (15). de Virgilio et al. (30) used real time bimolecular fluorescence complementation to visualize early interactions of Src after \( \alpha_{\text{IIb}}\beta_3 \) activation (i.e. Src-\( \alpha_{\text{IIb}}\beta_3 \) and Src-Syk interactions). Their study required stable association of Src with its interaction partners, whereas our approach (intermolecular FRET) allows complex re-dissociation together with higher temporal resolution of imaging (5 s versus 1 min).

Dynamic FRET specific for wild-type Src and Csk interactions was detected directly on the adhesion plane in cells plated on fibrinogen. Src-YFP and Csk-CFP were expressed in physiological concentrations, and the detected FRET efficiencies of 25–30% are in accordance with intramolecular FRET signals between CFP- and YFP-labeled proteins. This observation was confirmed by acceptor photobleaching, the classical FRET control experiment. Altogether, these data clearly demonstrate that the process of Src inactivation in living cells can be visualized by FRET.

By focusing on lamellipodia movements we could follow the dynamic of Src signaling and cytoskeletal rearrangements. Our data indicate that Src is periodically inactivated (every 2–3 min) by Csk recruitment at the leading edges.

The lamellipodia protrusion is then suppressed because of inhibition of actin filament polymerization, and the Src-Csk complex is moving inward leading to restart integrin signaling and protrusion by actin polymerization. Inward moving complexes transported by F-actin fragmentize and then form or are incorporated into focal adhesions as shown by our co-immunostaining experiments with vinculin (supplemental Fig. 3). A similar effect was described in A5-CHO cells co-expressing fluorescence-labeled c-Src and Syk, a downstream target of Src. After plating the cells on fibrinogen, complexes of c-Src and Syk moved inward in the cell and were observed in membrane ruffles and focal complexes (30).

This lamellipodial extension in spreading and migrating cells has already been studied in detail by Giannone et al. (32). In their study, they calculated a mean speed of 50 nm/s for actin wave movements in the initial phase of spreading. This is in good agreement to our measured Src-Csk complex velocity of 90 nm/s.

A recent review has summarized the role of Src and Csk in focal adhesions and their involvement in metastasis (10). Src activity was accompanied by reduced cell attachment and a deficient number of focal adhesions required for migration/invasion or cell detachment and proliferation. In contrast, Csk activity is enhanced for stable focal contacts when no mobility is required. This is underlined by our results showing FRET signals in focal adhesions of spreading, nonmigrating cells and is supported by our Src and Csk mutant studies.

Cells expressing constitutively active Src (SrcY529F-YFP) show only a few, very faint and unusually large focal contacts. These changes in focal adhesion structure are most likely because of increased integrin clustering and bundling of actin filaments. FRET signals were not detected in these adhesions confirming that Csk binding to Src requires Tyr-529. Cells expressing a kinase-inactive Csk (CskK222R-CFP) show effective adhesion. However, the focal contacts appear to be affected. Focal adhesions, usually flanking lamellipodia and causing the characteristic shape (28), are missing in these cells, thus resulting in a single extended and unstructured lamellipodium. A reduced attachment of the focal adhesions is visualized by the increased tendency of the leading edges to protrude. A decreased inactivation of Src at the leading edges (low FRET signal) supports this migration behavior. As Src inactivation is abated throughout the whole cell, there is no net migration visible, except for a widespread flattening morphology of the cells.

With regard to the role of Src and Csk in \( \alpha_{\text{IIb}}\beta_3 \)-dependent regulation of the cytoskeleton, our mutant experiments provided further insights. In the presence of constitutively active Src (SrcY529F-YFP), the cytoskeleton was apparently hyperactivated and the cortical cytoskeleton formed large membrane blebs, a typical dynamic seen when the membrane detaches from the cortex (33). Increased filopodia formation was observed in the presence of CskK222R-CFP, as well as Csk mislocation at the interface between lamellipodia and lamellae.

Interestingly, a distinct FRET signal was seen between wild-type Src-YFP and inactive CskK222R-CFP. According to kinetic studies by Lieser et al. (9) Csk binding to Src is described by a biphasic mechanism. The first step is described by a weak binding of Csk to Src accompanied by a slow conformational change (chemical clamp). Once the enzyme is locked into the catalytic cycle, phosphorylation takes place followed by the release of phospho-Src.

The Csk inactive mutant (CskK222R) is only “dead” in terms of kinase activity and SrcY529 phosphorylation. However, CskK222R still binds to Src by a chemical clamp as seen in the immunoprecipitation experiments with integrin \( \beta_3 \) (Fig. 2E, 4th lane) and therefore a FRET signal is observed. Interestingly, the Src_wt-YFP/CskK222R-CFP complex accumulates at the leading edges without inward movement assuming that phosphorylation of SrcY529 by Csk is important for the active rearward movement of the complex along the actin bundles to focal contacts.

Taken together, our experiments clearly demonstrate that a tight control of Src is essential for cytoskeletal regulation and reorganization. The results provide new information about the very important interaction of Src and Csk for platelet \( \alpha_{\text{IIb}}\beta_3 \) signaling and endothelial function.

The intermolecular FRET approach is a sensitive and efficient reporter of Src inactivation in living cells, and in this
respect could also be extended to other SFKs to define their role(s) in pathways controlled by β₁/β₂ integrins and other receptors.

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