

Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer

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Summary

Microscopy-based fluorescence resonance energy transfer (FRET) provides an opportunity to monitor molecular processes in the natural environment in live cells. Here we studied molecular interactions and tyrosine phosphorylation of paxillin, Crk-associated substrate (CAS), and focal adhesion kinase (FAK) in focal adhesions. For that purpose, these focal adhesion phosphoproteins, fused to cyan or yellow fluorescent proteins (CFP or YFP) were expressed in cultured fibroblasts. To assess the dynamics of tyrosine phosphorylation we used YFP- or CFP-tagged SH2 domain of pp60^{src} (dSH2), which specifically binds to phosphotyrosine residues. FRET measurements, combined with immunolabeling with phosphospecific antibodies revealed that FAK, CAS and paxillin are tyrosine phosphorylated in early matrix adhesions and that FAK is in FRET proximity to CAS and paxillin in focal complexes and focal adhesions. Data

suggest that paxillin incorporation into nascent focal complexes precedes its tyrosine phosphorylation, which then gradually increases. In cells treated with Rho-kinase inhibitors or expressing constitutively active Rac, focal complexes showed similar levels of paxillin tyrosine phosphorylation as seen in mature focal adhesions. Dynamic FRET-based examination indicated that paxillin phosphorylation occurs in specific areas (hotspots) within focal adhesions, whereas FAK phosphorylation is broadly distributed.

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Key words: Focal adhesions, FRET, Paxillin, FAK, CAS, Phosphorylation

Introduction

Integrin-mediated adhesion to the extracellular matrix (ECM) plays a pivotal role in the regulation of tissue integrity, cell migration and transmembrane signaling. These adhesions are formed in specialized sub-cellular sites, known as focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001) where matrix-attached integrin receptors interact with the actin cytoskeleton via a submembrane, multimolecular plaque. Many of the known plaque proteins contain binding sites for multiple (sometimes, over ten) partner molecules (Zamir and Geiger, 2001). For example, paxillin, one of the most prominent plaque proteins, can bind directly to integrins, vinculin, Crk, actopaxin, FAK and more (Turner, 2000a; Turner, 2000b). Based on extensive biochemical studies, models of 'interaction networks' of focal adhesions were proposed, where the potential intermolecular interactions were depicted (Zamir and Geiger, 2001). However, the hierarchy of these multiple interactions and the mechanisms regulating their formation and turnover in the living cell are still poorly understood.

One of the mechanisms regulating the formation and dynamics of focal adhesions involves tyrosine phosphorylation. Focal adhesions contain several tyrosine-specific kinases and phosphatases, as well as their substrates (Geiger et al., 2001) whose phosphorylation creates docking

sites for phosphotyrosine (PY) binding domains [e.g. Src homology-2 (SH2)] on appropriate partner molecules (Pawson et al., 2001). The main kinases responsible for these phosphorylation events are FAK and pp60^{src}, whose absence or inhibition affects focal adhesion dynamics (Ilic et al., 1995; Richardson and Parsons, 1996; Volberg et al., 2001) and adhesion-mediated signaling (Schaller, 2001). The major substrates of these enzymes are the adapter proteins paxillin, CrkII and CAS, as well as Src and FAK themselves. The phosphorylation of these proteins can generate docking sites for SH2-containing adapter and signaling molecules (Bellis et al., 1995; Harte et al., 1996; Panetti, 2002; Schaller and Parsons, 1995), and thereby affect focal adhesion composition and dynamics. Paxillin phosphorylation is induced by FAK and Src upon stimulation with growth factors (Abedi et al., 1995; Rankin and Rozengurt, 1994) or activation of Rho A (Barry and Critchley, 1994; Flinn and Ridley, 1996). Tyrosine phosphorylation of CAS is mediated by Src upon complex formation with FAK (Ruest et al., 2001) and its localization to focal adhesions is largely dependent on its Src binding and its SH3-mediated interaction with FAK (Nakamoto et al., 1997). To date, however, only little is known about the formation and dynamics of Paxillin-CAS-FAK complexes in live cells.

In this study, we used fluorescence resonance energy transfer (FRET) measurements to probe tyrosine phosphorylation and

the consequent molecular interactions between the focal adhesions proteins vinculin, paxillin, p130Cas and FAK in live cells. For this purpose we expressed different combinations of these focal adhesions molecules, fused to cyan and yellow fluorescent proteins (CFP and YFP) as FRET donor and acceptor pairs. As a probe for phosphotyrosine (PY) we used CFP or YFP, fused to a tandem repeat of the SH2 domain (dSH2) of pp60^{src}, which specifically binds to tyrosine-phosphorylated sites on proteins associated with focal adhesions (Kirchner et al., 2003). The SH2 domain of Src was shown to have a broad specificity to tyrosine-phosphorylated targets (Nollau and Mayer, 2001) and to localize to focal adhesions (Kirchner et al., 2003). Analyses of cells expressing dSH2-GFP showed that at low expression levels the intensity of this probe is linearly correlated with the density of tyrosine-phosphorylated sites, detected by antibody labeling. High expression of dSH2 induced elevated tyrosine phosphorylation, probably by interfering with dephosphorylation (Kirchner et al., 2003).

Our primary approach for measuring molecular proximities is microscope-assisted FRET, based on the stimulation of acceptor fluorescence emission. It has been shown that FRET is a powerful approach for measuring molecular proximities at a range of ~10 nm and is thus capable of exploring protein-protein interactions (Jares-Erijman and Jovin, 2003; Miyawaki and Tsien, 2000). FRET is based on the principle that two close fluorophores with overlapping excitation and emission spectra, interact via dipole-dipole coupling, causing the excitation energy of the 'donor' to be transferred to the 'acceptor', resulting in light emission as longer-wavelength fluorescence (Jares-Erijman and Jovin, 2003; Miyawaki and Tsien, 2000). Here we present, for the first time, data on the dynamics of tyrosine phosphorylation of paxillin, CAS and FAK in focal adhesions and focal complexes of live cells. We show that a large fraction of the three proteins is tyrosine phosphorylated in focal complexes and remains phosphorylated during focal adhesion formation. The phosphorylation state of paxillin in focal complexes, induced by active Rac1, is similar to the phosphorylation state in focal adhesions induced by RhoA. The three major tyrosine-phosphorylated proteins in adhesion sites, CAS, paxillin and FAK colocalize in focal complexes and focal adhesions, and FAK displays FRET proximity to both CAS and paxillin. Taken together, these data map molecular proximities in adhesion complexes of live cells shedding light on both PY-dependent and PY-independent protein associations during focal adhesion development.

Results

FRET analysis of protein tyrosine phosphorylation in living cells

Vinculin and paxillin are two prominent focal adhesion components that were shown to interact *in vitro* (Turner et al., 1990; Wood et al., 1994). In contrast to vinculin, paxillin contains multiple tyrosine-phosphorylation sites, of which four (PY31, PY40, PY118, PY181) were shown to be prominent SH2-binding sites (Brown and Turner, 2004; Nakamura et al., 2000). As previously described (Kirchner et al., 2003), SH2-binding sites can be visualized *in vivo*, using GFP fused to a tandem repeat of the SH2 domain of Src. In order to map the PY sites relative to total paxillin and vinculin, donor-acceptor pairs of either of the two proteins and of dSH2, were expressed

in NIH 3T3 cells. Intensity ratio images revealed an extensive spatial overlap between dSH2 and each of the two proteins, though dSH2 intensity was not always correlated with high vinculin or paxillin fluorescence (Fig. 1A).

FRET, expressed as the 'FRET index' (FI) (see Materials and Methods) for paxillin and dSH2 was usually very high, reaching average FI values of >30.0 (Fig. 1A,B). This was the case when paxillin was expressed as donor (fused to CFP) and SH2 expressed as acceptor (fused to YFP) and vice versa. These results indicate that tyrosine-phosphorylated residues are located at 'FRET range' from the paxillin-fused fluorophores. To biochemically probe the interaction of paxillin with the dSH2 reporter we immunoprecipitated YFP-dSH2 and showed that a large number of tyrosine-phosphorylated proteins, including paxillin, were co-immunoprecipitated (Fig. 1C). In contrast to the high FI values for paxillin and dSH2, the values obtained for the vinculin-dSH2 pair, were low (average FI<5) irrespective of which protein was selected as donor and acceptor (Fig. 1A,B), suggesting that there were essentially no available SH2-docking sites on vinculin or within FRET range from it. This was further corroborated by immunoprecipitation experiments, showing that vinculin fails to co-precipitate with dSH2 (see Fig. S1 in supplementary material).

To assess directly the FRET efficiency, as measured by sensitized emission, we used an alternative approach for quantifying FRET, namely, the 'dequenching of the donor' following acceptor photobleaching (Siegel et al., 2000). Photobleaching of the YFP-dSH2 induced a 7% increase of CFP-paxillin fluorescence (Fig. 2), but had no significant effect on CFP-vinculin fluorescence (data not shown).

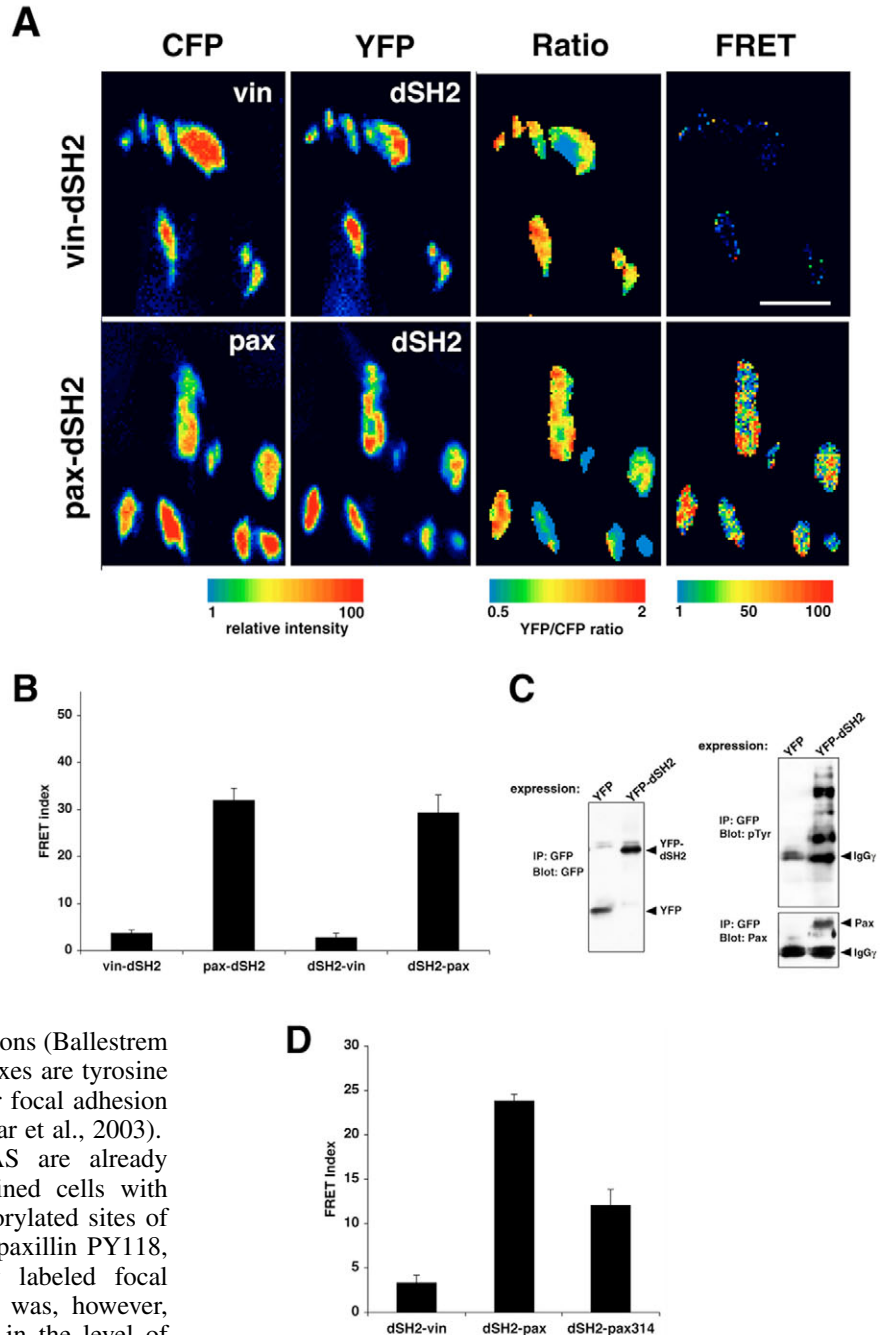
To test whether the high paxillin/dSH2 FRET values depend on direct phosphorylation of the fluorescently-tagged paxillin, we performed FRET experiments with cells expressing a truncated YFP-paxillin construct devoid of the major PY sites at positions 31, 40, 118, 181 (denoted pax Δ 1-314) and CFP-dSH2. FI values in these experiments were about half of those detected with full-length paxillin. It is noteworthy that these values were still significantly higher than those obtained with the dSH2/vinculin pair (Fig. 1D), suggesting that the measured paxillin-dSH2 FRET was partly attributable to intramolecular cross-talk and partly to FRET between paxillin and tyrosine phosphorylated proteins located near by.

FI measurements between dSH2 and focal adhesion components were extended to other tyrosine-phosphorylated components of the submembrane plaque, including FAK and CAS. For that purpose we expressed each of these molecules fused to YFP, with dSH2-CFP and measured the FI. High FI values for both dSH2-FAK and dSH2-CAS were obtained (Fig. 3). These values were similar or even higher than those measured for dSH2-paxillin (Fig. 3B). Intensity ratio images of dSH2/FAK and dSH2/CAS showed a largely homogeneous distribution, indicating their almost identical localization in focal adhesions. Moreover, both molecules co-immunoprecipitated with dSH2-CFP (Fig. 3C), indicating that both FAK and CAS can associate with dSH2.

Tyrosine phosphorylation of paxillin, CAS and FAK in focal complexes

During cell migration, small dot-like adhesions, known as focal complexes, form at the leading edge. Some of these adhesions

Fig. 1. Tyrosine phosphorylation of focal adhesion components. A construct consisting of two consecutive SH2 domains (dSH2) fused to CFP or YFP was used as a reporter for PY residues. For FRET measurements between PY and vinculin or paxillin, dSH2 (fused to CFP or YFP) was co-transfected with paxillin or vinculin, fused to the complementary fluorophore. (A) Single-color images, ratio images and FRET images are displayed for the indicated pairs. Note high CFP-paxillin/YFP-dSH2 (pax-dSH2) and low FRET for CFP-vinculin/YFP-dSH2 (vin-dSH2). Bar, 2 μ m. (B) Quantification of FI measurements with the indicated FRET pairs. The data are presented as mean \pm standard error of FI in focal adhesions of 15 to 32 cells. (C) Western blots showing, on the left, immunoprecipitation of YFP and YFP-dSH2 from cells expressing these constructs. The blot on the upper right shows that a large number of tyrosine phosphorylated proteins co-immunoprecipitate with the YFP-dSH2 domain expressed in NIH 3T3 cells. One of the proteins that co-precipitated is paxillin (lower right blot). No tyrosine-phosphorylated proteins co-precipitated from control lysates from NIH 3T3 cells expressing YFP only. (D) Quantification of FRET measurements with the indicated FRET pairs. Note that FRET between dSH2 and a truncated form of paxillin devoid of major tyrosine phosphorylation sites is reduced by half compared with FI values between dSH2 and full-length paxillin, but still significantly higher than between dSH2 and vinculin.



eventually grow and 'mature' into focal adhesions (Ballestrem et al., 2001; Clark et al., 1998). Focal complexes are tyrosine phosphorylated and contain paxillin and other focal adhesion components (Nakamura et al., 2000; Zaidel-Bar et al., 2003).

To test whether paxillin, FAK and CAS are already phosphorylated in focal complexes, we stained cells with antibodies directed against the major phosphorylated sites of these molecules. Antibodies directed against paxillin PY118, FAK PY397 and CAS PY165 all readily labeled focal complexes at the cell periphery (Fig. 4). It was, however, noticed that there was a large heterogeneity in the level of phosphorylation of these proteins in focal complexes. Moreover, the antibody directed against paxillin shows strong cytoplasmic staining, whereas the phosphospecific antibodies labeled mainly the adhesion structures (not shown). This suggests that these proteins become phosphorylated only after they become incorporated into adhesion complexes.

To study tyrosine phosphorylation of paxillin in live cells in real time, we plated NIH 3T3 fibroblasts, co-expressing CFP-paxillin and YFP-dSH2 on fibronectin and monitored the development of new adhesion sites under the advancing lamellipodium. The recruitment of paxillin to focal adhesions precedes the local increase in PY levels (Fig. 5) (see also Kirchner et al., 2003). As soon as we detected dSH2-YFP in focal complexes we were also able to detect modest, but significant FRET values that increased during transformation

of focal complexes to focal adhesions (Fig. 5A, see ellipse). Since values of intermolecular FRET depend on both distance and concentration of donor and acceptor fluorophores, the increase of FRET between paxillin and dSH2 could be attributed to changes in donor/acceptor ratio. Indeed, analysis of the fluorescence intensities, confirmed that the donor/acceptor ratio changes during the transition from focal complexes to focal adhesions, whereas intensities in mature focal adhesions remain rather constant over time (Fig. 5B). In all cases, the donor-acceptor combination and relative intensities were selected and optimized to provide images of adequate dynamic range and high signal-to-noise ratio.

To determine whether the changes in paxillin and PY in

Fig. 2. Paxillin proximity to PY sites, as detected by FRET measurements via acceptor photobleaching. (A) Cells expressing paxillin-CFP and dSH2-YFP were placed on an inverted microscope. For FRET measurements CFP and YFP images were captured before and after YFP photobleach. FRET efficiency (E%) was calculated pixel by pixel: 1- CFP before photobleaching and CFP after photobleaching. Note high FI values are seen in 'hotspots' of the total focal adhesion area. Bar, 4 μ m. (B) Left, focal adhesion donor (CFP) image before (red) and after (green) acceptor photobleaching. Right, a line scan through the focal adhesion images showing the increase in donor fluorescence as a result of the acceptor bleaching.

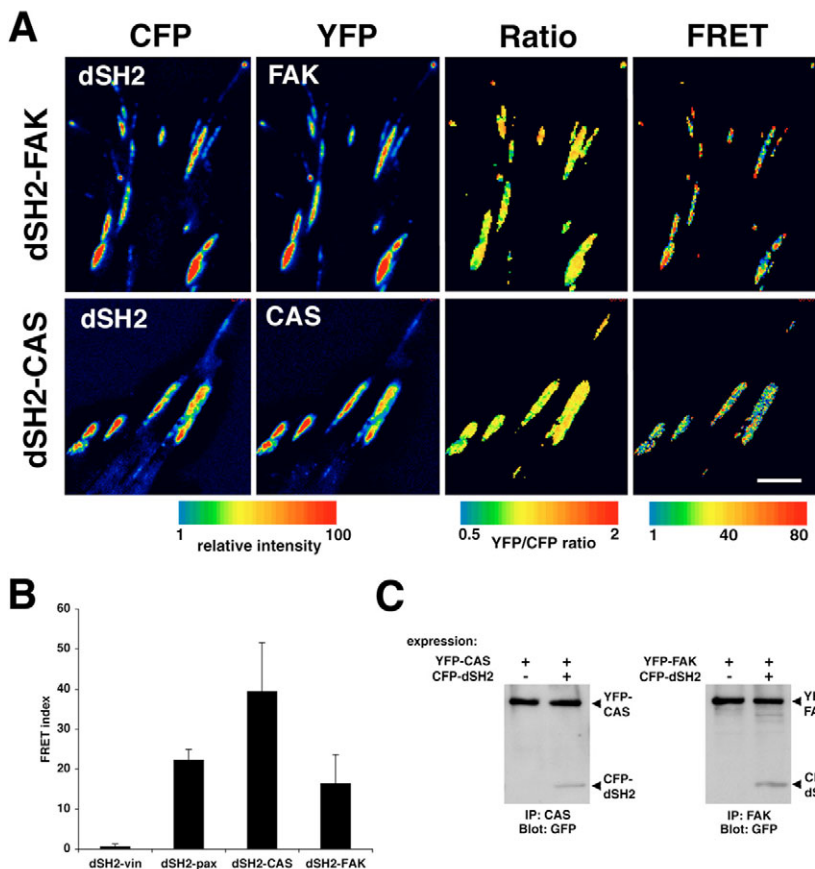
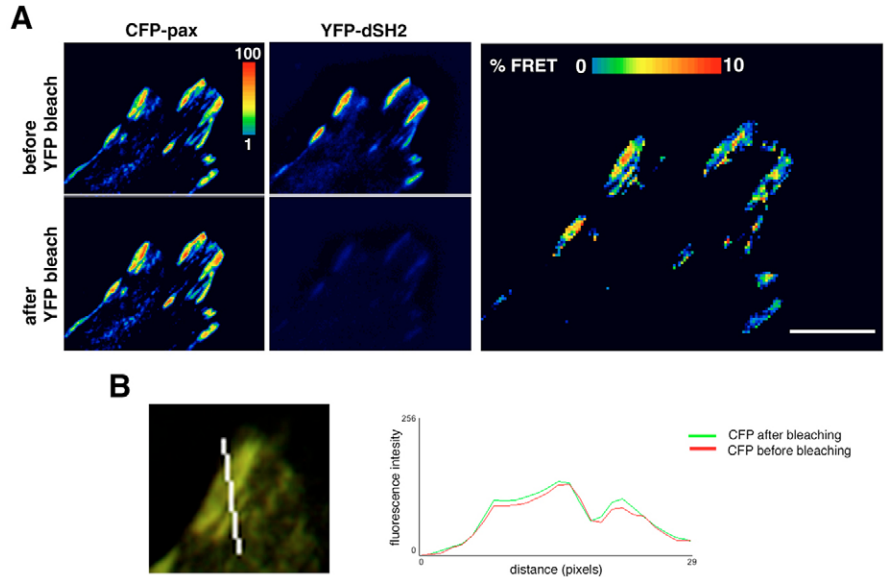


Fig. 3. FAK and CAS are in FRET proximity to PY-containing sites. (A) Single-color images, ratio images and FRET images are displayed for the indicated pairs. High FI values were detected for both dSH2-FAK and dSH2-CAS donor-acceptor pairs. Bar, 2 μ m. (B) Quantification of FRET measurements with the indicated FRET pairs. The data are presented as mean \pm s.e. of three experiments. (C) Immunoprecipitation of CAS from lysates of 293 cells expressing YFP-CAS together with CFP-dSH2 leads to the co-precipitation of CFP-dSH2 (left). CFP-dSH2 co-precipitates with FAK in immunoprecipitation experiments from lysates of 293 cells expressing YFP-FAK and CFP-dSH2 (right).

maturing focal complexes, depend on their transformation into definitive focal adhesions or are merely time dependent, we blocked focal adhesion formation using the ROCK inhibitor Y27632 (Narumiya et al., 2000). Treatment with this drug results in a massive accumulation of focal complexes at the cell periphery (Fig. 6A). The FI in focal complexes of Y27632-treated cells was comparable to that found in focal adhesions of control cells (Fig. 6A,C), suggesting that similar fractions of paxillin are surrounded by a comparable fraction of SH2-binding sites in the two structures. To compare FRET levels in focal complexes and in focal adhesions of the same cells, we expressed constitutively active Rac together with CFP-paxillin and YFP-dSH2 in NIH 3T3 cells. These cells show a rim of focal complexes at their periphery, as well as definitive focal adhesions at more central positions (Fig. 6B). The life span of focal complexes in these cells is about ten-fold longer (unpublished observation) than that of focal complexes found in the lamellipodia of untreated cells. Interestingly, FRET values obtained in these cells were similar for focal adhesions and focal complexes (Fig. 6). The higher FRET values in focal complexes of cells expressing active Rac, or treated with Y27632, compared with controls (see Fig. 5), could be due to a slower turnover of proteins such as paxillin in focal complexes promoting the accumulation of SH2-binding sites or activation of signaling cascades that generate SH2 docking sites. In both cases we strongly influence 'inside-out' signaling cascades known to be involved in the formation of focal adhesions.

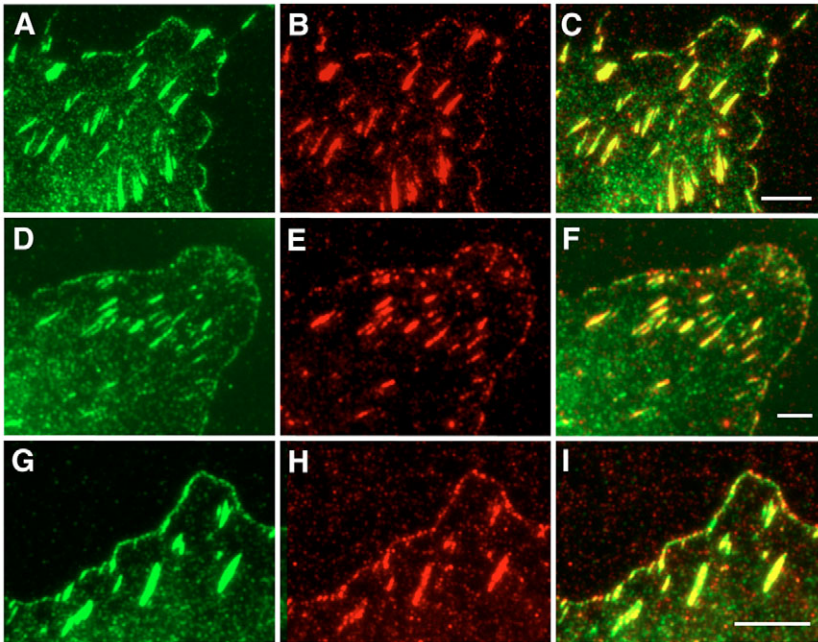
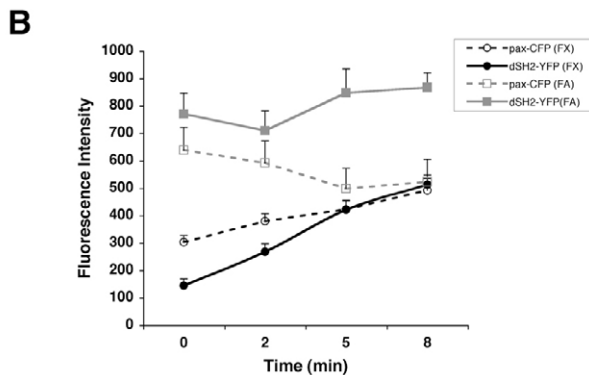
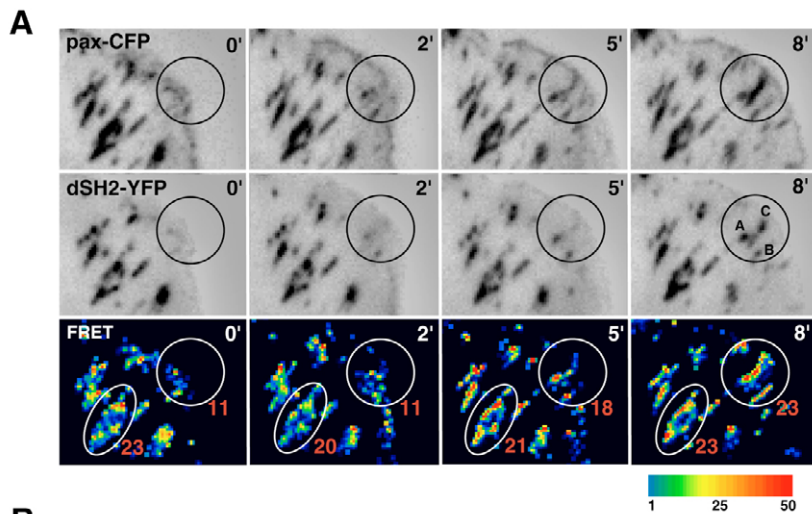


Fig. 4. Paxillin, CAS, and FAK phosphorylation in focal complexes and focal adhesions. NIH 3T3 cells were fixed and labeled for paxillin (A,D,G); phosphospecific antibody to paxillin PY118 (B); phosphospecific antibody against the FAK autophosphorylation site PY397 (E); phosphospecific antibody directed against CAS PY165 (H). Merged images are shown in the right-hand column (C,F,I). Note that paxillin, FAK and CAS are phosphorylated on the specific PY sites in focal complexes and focal adhesions. Bars, 5 μ m.

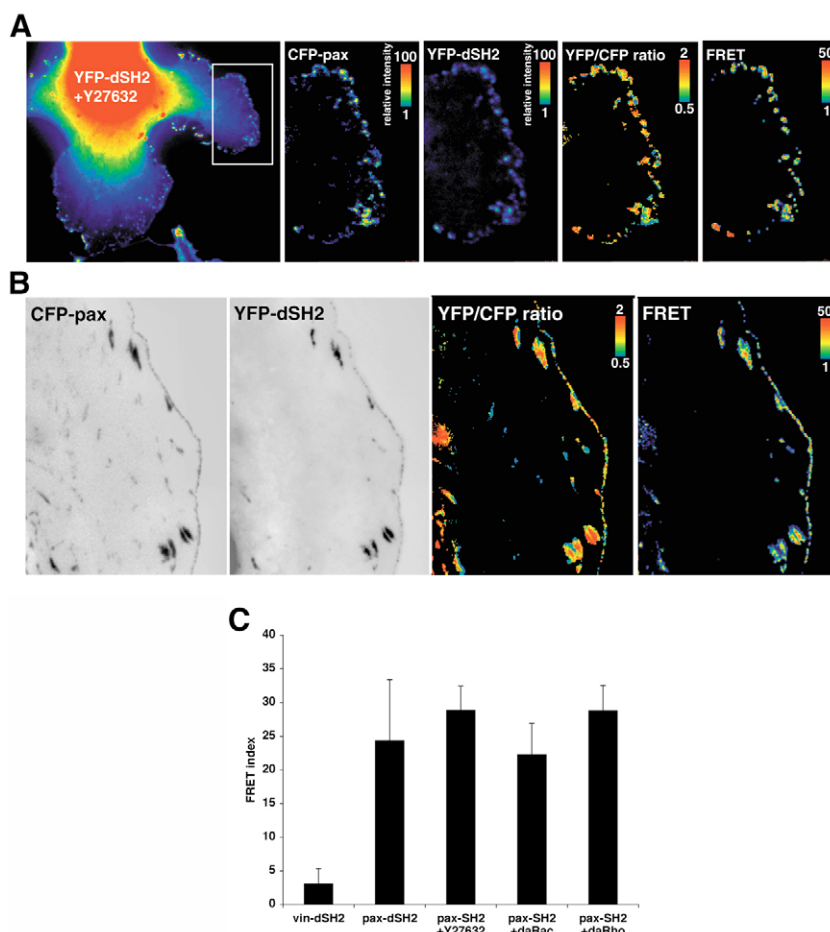


Molecular interactions between paxillin, FAK and CAS in mature focal adhesions FAK is a pivotal focal adhesion molecule, which is involved not only in tyrosine phosphorylation in these sites, but also provides docking sites for several focal adhesion molecules, including paxillin and CAS (Parsons et al., 2000; Schaller and Parsons, 1994). Indeed, FAK, CAS and paxillin co-localize within focal adhesions and focal complexes in NIH 3T3 cells (Fig. 7, see also Fig. 4). Ratio images of CFP-FAK and YFP-CAS indicate an almost identical distribution of the two proteins in mature focal adhesions, whereas the distribution of paxillin and FAK is somewhat less overlapping, suggesting an enrichment of FAK in discrete areas of focal adhesions, compared with paxillin, which is more widely distributed (Fig. 7A). FRET values for FAK-CAS and FAK-paxillin were high in both mature focal adhesions and in focal complexes ($FI > 16$; Fig. 7A,B,C). Although we detected colocalization of CAS with paxillin (Fig. 4), we were not able to measure FRET between them because overexpression of paxillin apparently inhibited CAS localization to focal adhesions, rendering FRET measurements unreliable, owing to high differences in fluorescence intensity (paxillin \gg CAS) in focal adhesions (not shown).

Taken together, these FRET data, showing close association of FAK with paxillin and CAS in focal complexes, suggest that phosphorylation of the two proteins via FAK

Fig. 5. Paxillin phosphorylation during focal adhesion development. (A) First two rows of the time-lapse recording show localization of CFP-paxillin and YFP-dSH2 along the lamellipodium of NIH 3T3 fibroblast; for better visualization black and white inverted-contrast images are shown. The third row shows calculated FI between CFP-paxillin and YFP-dSH2. Note that during the development of focal adhesions, paxillin accumulation precedes the increase in PY levels (compare encircled areas in rows one and two). FRET values of focal complexes in the encircled area increase steadily until reaching a level of $FI > 20$, as in mature focal adhesions. High FRET values in mature focal adhesions (ellipse) remained at $FI > 20$ during the entire period of time-lapse recording. (B) Measurements of fluorescence intensity maxima during transition of focal complexes to focal adhesions (black lines; encircled area in A) compared with those in focal adhesions (grey lines; ellipse area in A) during the period of observation. Note that during the transition of focal complexes to focal adhesions, acceptor fluorescence intensity, although initially below donor intensity, rises above the donor intensity at 8 minutes. In focal adhesions, acceptor intensity remains in excess of the donor intensity.

Fig. 6. Paxillin phosphorylation in focal complexes and focal adhesions. (A) Single-color images, ratio and FRET image of CFP-paxillin and YFP-dSH2 in NIH 3T3 cells treated with the ROCK inhibitor Y27632 to induce accumulation of focal complexes. Note the high FI in focal complexes between CFP-paxillin and YFP-dSH2 indicating high tyrosine phosphorylation of paxillin in these structures. (B) A cell expressing a dominant-active form of Rac in addition to the donor-acceptor pair paxillin-dSH2. Note the co-existence of focal complexes at the cell periphery and the larger focal adhesions behind the cell edge. Single-color images of CFP-paxillin and YFP-dSH2 are presented as inverted black and white images for better visualization. High FI values in focal adhesions and focal complexes suggest that a large and comparable fraction of paxillin is tyrosine phosphorylated in both structures. (C) Quantification of FRET between the indicated donor-acceptor pairs in focal adhesions, in focal complexes of Y27632-treated cells, in focal adhesions plus focal complexes in cells expressing dominant-active Rac1 (daRac) and in focal adhesions of cells expressing active RhoA (daRho). The data are presented as mean \pm s.e. of FI in focal adhesions or focal complexes measured in five to ten cells.



occurs at early stages of focal adhesions maturation. The low amount of CAS fluorescence in paxillin co-expressing cells might be due to a competition of the two on the binding to FAK.

Phosphorylation dynamics in focal adhesions

The direct measurement of protein incorporation and tyrosine phosphorylation in focal adhesions of live cells allowed us to probe the dynamics of these processes. For these experiments we chose better probe combinations to minimize photobleaching effects. Intensity ratios and the corresponding FRET images of the same temporal series of paxillin-dSH2 and FAK-SH2 pairs were recorded at one-minute intervals over a period of 4 minutes (Fig. 8). To reduce noise, pixels were smoothed in these images to a kernel size of 2.

Intensity ratios between paxillin and dSH2, revealed local variations, however, the overall pattern remained rather stable over a period of several minutes (Fig. 8). As shown above (Fig. 1), areas with especially high concentrations of PY were found within homogeneously distributed paxillin-rich areas. Regions with high dSH2-to-paxillin ratios usually also displayed higher FI values (Fig. 8; supplementary material Fig. S2), suggesting that there are relatively stable 'hot spots' with high kinase activity within the focal adhesions. For the FAK-dSH2 pair, ratio images were more homogeneous owing to the high spatial correlation between the localization of the two in the focal adhesion area. FI values were usually high throughout the

overlap area of the two probes during the entire recording time (Fig. 8). This suggests that FAK localizes in specific areas of focal adhesions where it undergoes auto-phosphorylation and further phosphorylates its major targets including paxillin and CAS.

To explore the functional significance of the highly phosphorylated 'hot spots' within focal adhesions we have used time-lapse video microscopy of cells expressing dSH2-YFP to monitor local changes in focal adhesions structure. Such observations suggest that highly phosphorylated areas of focal adhesions are particularly dynamic and tend to undergo turn-over and disassembly (Fig. 9). Since long-term FRET imaging of live cells is not compatible with cell survival and growth, more extensive studies will be required to address this question using combined transmitted light/fluorescence movies of live cells.

Discussion

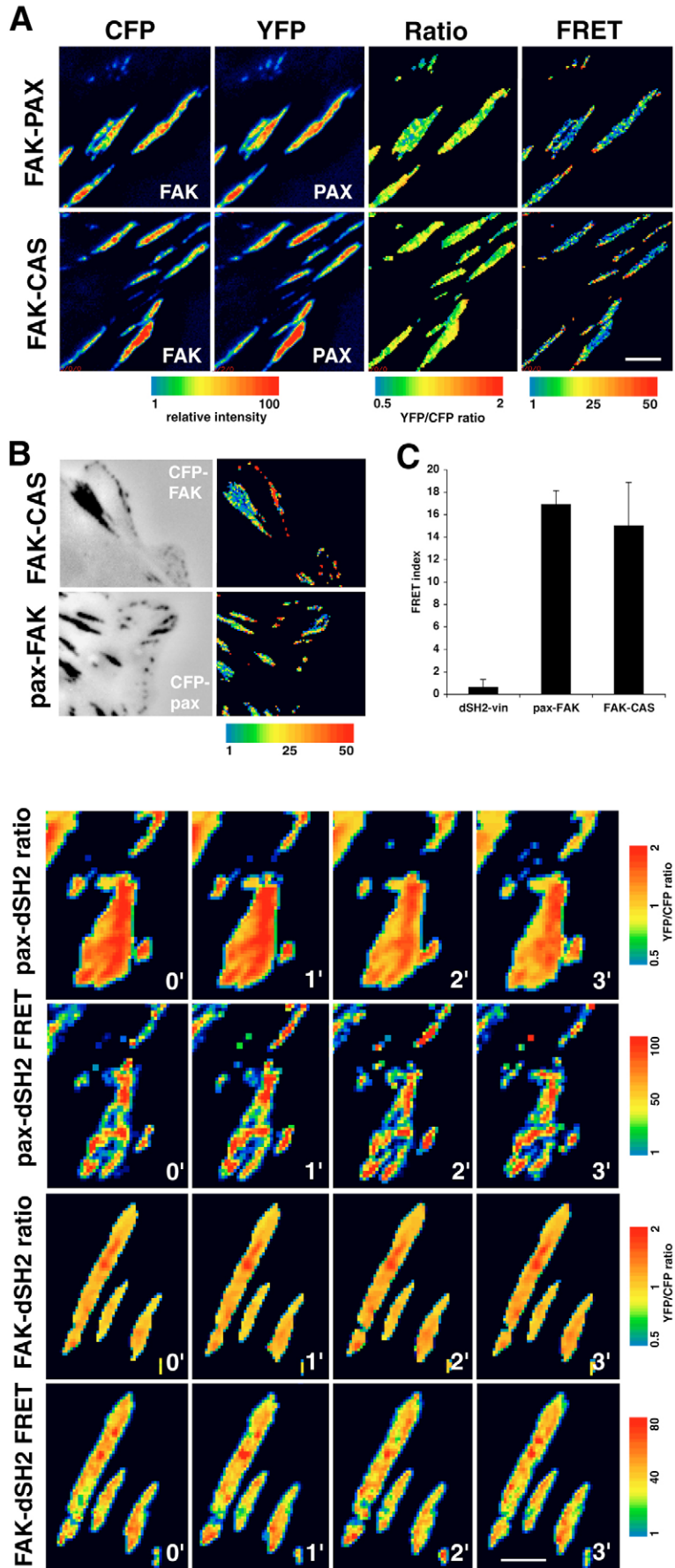
The use of FRET enables the mapping of intermolecular distances of a few nanometers between a donor and an acceptor fluorophore (Jares-Erijman and Jovin, 2003; Miyawaki and Tsien, 2000) shedding light on the formation of molecular complexes both in vitro and in vivo. In this study we have applied microscopy-based FRET measurements for studying molecular interactions in cell-matrix adhesion complexes. This microscopy-based measurement in live cells has temporal and spatial resolution lacking from biochemical data. Specifically

Fig. 7. FRET for FAK together with either paxillin or CAS. NIH 3T3 cells were transfected with cDNA encoding pairs of CFP- and YFP-fusion proteins as indicated. (A) Images are displayed in spectral scale for the CFP and YFP fusion proteins, for their fluorescence ratio, and for the calculated FRET between the co-expressed molecules. CFP-paxillin/YFP-FAK (pax-FAK) and CFP-FAK/YFP-CAS (FAK-CAS) pairs display relatively high FRET values. (B) FRET of indicated donor/acceptor pairs in focal complexes. First image displays the localization of the acceptor in inverted grey levels (C) Quantitative FRET analysis of the indicated pairs. The data are presented as mean \pm s.e. of FI in focal adhesions measured in 8 to 15 cells. Bar, 2 μ m.

it has the spatial resolution to detect the molecular proximity to phosphorylation sites in individual focal adhesion of individual cells, or even subdomains of these adhesion sites. This information can be retrieved continuously, thus detecting local or global changes and fluctuations. Given that such processes are not synchronized in different cells or cellular regions, this spatio-temporal information would be missed by a biochemical approach.

It is noteworthy that FRET data provide high-resolution information about molecular distances between the labeled fluorophores, but cannot prove direct binding between the modified proteins. This limitation is particularly significant when the donor and acceptor fluorophores are large molecules whose exact position, relative to the respective fusion partner is usually poorly defined. Nevertheless, high FRET values for the dense aggregation of components at adhesion sites reflect average proximities that sets spatial and dynamic constraints on the multi-protein complex assembly. For example, the fact that FRET between wild-type paxillin and dSH2 is significantly higher than that obtained with the paxillin mutant lacking a major phosphorylation site strongly suggests that direct molecular interactions have a major contribution to the measured FRET values. Furthermore, all proteins tested that are known to be tyrosine phosphorylated (i.e. paxillin, FAK and CAS) show high FI values with the dSH2 probe whereas proteins that are devoid of such sites [i.e. vinculin (Fig. 1) zyxin and actin], did not show significant FI levels, despite extensive spatial overlap at the resolution of the microscope. The direct interaction of paxillin, CAS and FAK (but not vinculin) with the dSH2 probe is also corroborated by the co-

Fig. 8. Dynamic changes of fluorescence intensity ratio and of FRET of paxillin-dSH2 and FAK-dSH2 pairs. The fluorescence measurements were taken at 1-minute intervals. The color scales on the right indicate the fluorescence ratio or FI values. Note that the temporal variation in the ratio of both donor/acceptor pairs is relatively stable. FI values for the FAK-dSH2 pair showed some local fluctuations, whereas high values for paxillin-dSH2, mostly restricted to areas with high PY remained stable over time. Bar, 2 μ m.



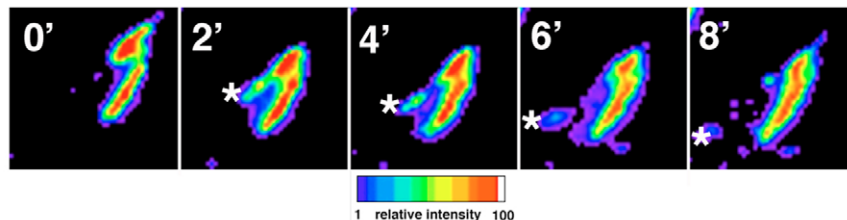


Fig. 9. A highly tyrosine phosphorylated focal adhesion at the retracting edge of a cell displays a dynamic reshaping, including a disconnected region (area indicated by asterisk) that moves towards the cell center.

immunoprecipitation experiment. It is interesting to note the high ‘self-FRET’ values obtained at focal adhesions with the pair dSH2-CFP/dSH2-YFP (supplementary material Fig. S3). This phenomenon is attributable either to the presence of clustered phosphorylated sites on the same molecules (Panetti, 2002), or on closely interacting ones. The dynamic analysis described here indicates that local PY levels located at FRET range from paxillin, gradually increase during focal adhesions development from focal complexes, either by the local phosphorylation of the different plaque proteins, or by the continuous recruitment of phosphorylated molecules to these sites.

Our main objective in this study, was to elucidate the spatial and temporal relationships between the major tyrosine phosphorylated focal adhesions molecules, paxillin, CAS and FAK and characterize their phosphorylation during focal adhesions formation and maturation. The phosphorylation of these proteins triggers their interaction with several SH2-bearing (mostly tyrosine-phosphorylated) partners (O’Neill et al., 2000; Panetti, 2002; Schaller, 2001; Turner, 2000b) that further regulate cell adhesion and migration. Using FRET experiments combined with immunolabeling for phosphorylated paxillin (PY 118), FAK (PY397) and CAS (PY 165), using phospho-specific antibodies, we show that tyrosine phosphorylation of these proteins occurs in focal complexes along the lamellipodium, and remains high during transition of focal complexes into focal adhesions. We were not able to see a clear hierarchy in the phosphorylation status of the three proteins. However, it was reported that phosphorylation of CAS and paxillin occurs via Src binding to phosphorylated FAK at position PY397 (Ruest et al., 2001; Vuori et al., 1996) suggesting that FAK auto-phosphorylation might occur first. It is noteworthy that high FAK-paxillin and FAK-CAS FRET is already detected in focal complexes, suggesting that both the molecular interactions and phosphorylation of FAK, CAS and paxillin occur at an early stage of adhesion. We were unable to reliably measure FRET between CAS and paxillin because overexpression of the latter resulted in a marked reduction in CAS fluorescence in focal adhesions (not shown). Although it is known that CAS and paxillin bind to different domains on FAK we speculate that these two proteins actually compete for binding to FAK.

Another feature offered by quantitative microscopy-based analysis and FRET is the possibility to visualize and measure dynamic molecular events. In this study we show that in stationary focal adhesions, the areas of high phosphorylation maintaining high FRET relationships with paxillin are rather stable over time. The regions with the highest FI values were those that also contain the highest levels of the PY probe dSH2. Paxillin, on the other hand, was more widely distributed within the focal adhesion. Interestingly, FAK and dSH2 distributed

almost identically in focal adhesions, which suggests that FAK-containing ‘hotspots’ define sub-domains within focal adhesions, exhibiting a particularly high density of SH2-binding sites.

Another intriguing aspect of this study is the functional significance of the tyrosine phosphorylation hotspots in focal adhesions. Time-lapse experiments using cells expressing the dSH2 domain suggest that these hotspots may play a role in stimulating focal adhesion turnover. This is in line with a previous report showing that FAK- or Src-null cells form abnormally stable focal adhesions, whereas expression of deregulated Src greatly increases focal adhesion turnover (Katz et al., 2003). This phenomenon can be attributed, at least in part, to the activation of the Rho-family GTPase Rac1 following phosphorylation-dependent activation of the CAS-DOCK180/ELMO (Zaidel-Bar et al., 2005) or the Tiam1 (Erez et al., 2005; Mertens et al., 2005) exchange factor systems, eventually leading to dispersal of focal adhesions.

In conclusion, the findings described in this article shed new light on the molecular and functional diversity of adhesion complexes. Earlier studies showed that different forms of integrin-mediated adhesions (e.g. focal adhesions, focal complexes and fibrillar adhesions) display distinct molecular composition, morphology and subcellular distribution and dynamics (Ballestrem et al., 2001; Zaidel-Bar et al., 2003; Zamir et al., 1999). Furthermore, even individual adhesion sites were often shown to be non-homogeneous with regards to their molecular composition, with distinct ‘sub-domains’, displaying distinct molecular characteristics (Kirchner et al., 2003; Zamir et al., 1999). The significance of these variations is still poorly understood, and further studies addressing the underlying functional significance are currently underway. In this work we show another level of molecular diversity within focal adhesions, manifested by the presence of ‘hot-spots’ of high local levels of tyrosine phosphorylation within the adhesion sites. In view of the central importance of tyrosine phosphorylation for focal adhesion assembly, turnover and signaling, the molecular mechanisms regulating the formation and dynamics of these phosphorylated sub-domains may play a major role in controlling cell migration, matrix attachment and adhesion-mediated signaling.

Materials and Methods

Cells and reagents

NIH 3T3 cells were cultured in DMEM, supplemented with antibiotics and 10% FCS (complete medium). Transient transfections were performed with LipofectAMINE PLUS (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. YFP- and CFP-actin constructs were derived from GFP-actin (Choidas et al., 1998). Paxillin cDNA (kindly provided by K. Yamada, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) and vinculin cDNA were cloned into ECFP-C1 and EYFP-C1 (Clontech, Palo Alto, CA). CFP- and YFP-FAK was obtained from R. Horwitz (University of Virginia, School of Medicine, VA) and YFP-p130Cas was a gift from A. Bouton (University of West Virginia,

Morgantown, WV). The PY reporter used here consisted of two repeats of the SH2 domain of pp60^{src} (dSH2), fused to YFP or CFP as described elsewhere (Kirchner et al., 2003). The transfection conditions were selected so that the expression level of the acceptor fluorophores (YFP-tagged proteins) over the donor (CFP-tagged proteins) was kept between 2:1 and 1:2 in focal adhesions, to minimize the bleed-through corrections in FI (see below). For FRET imaging, cells expressing donor and acceptor proteins for 24 hours were plated in serum-free F12 medium, buffered with 25 mM HEPES, on fibronectin-coated (20 µg/ml) glass-bottom dishes (MatTek corporation, Ashland, MA).

Digital microscopy

Images were recorded on an Axiovert 100 TV inverted microscope (Zeiss, Oberkochen, Germany) equipped with an incubation chamber, a 100 W mercury lamp, a 100×/1.4 oil Plan-Apochromat objective (Zeiss, Oberkochen, Germany), excitation and emission filter wheels and a CCD Camera (CH300/CE 350, Photometrics, Tucson, AZ) with KAF1400 CCD chip, controlled by a DeltaVision system (Applied Precision, Issaquah, WA). The filters used were for the CFP channel: 29379 excitation filter (Chroma, VT), 455DCLP dichroic mirror, and F32-044 emission filter (AHF Analysentechnik, Tübingen, Germany); for the FRET channel (CFP excitation and YFP emission): 29379 CFP excitation filter (Chroma), 455DCLP dichroic mirror (AHF Analysentechnik) and 27203 YFP emission filter (Chroma); and for the YFP channel: 24000 excitation filter with a BS28311 dichroic mirror and the 27203 emission filter (Chroma). For FRET analysis a sequence of three images was recorded every minute, for 6–10 minutes, starting with the CFP channel, then the FRET, and finally the YFP. The three images were acquired within less than 5 seconds, a time sufficiently short to make any adhesion structures motion negligibly small, as established by second imaging at the end of the sequence the first color. In every session 'bleed-through' coefficients of YFP and CFP in the FRET channel (corresponding to α and β in the equation below) were determined (Zamir et al., 1999).

To measure FRET via de-quenching of CFP, photobleaching of YFP was performed using a D535/50 emission filter, 565DLCP beamsplitter, and E590LP emission filter (all AHF Analysentechnik). For FRET measurements CFP image was acquired before and after YFP photobleaching. FRET efficiency (E%) was calculated: $E\% = (1 - \text{CFP before photobleaching} / \text{CFP after photobleaching}) \times 100$.

Image analysis

FRET analysis was performed as previously described (Kam et al., 1995) with slight modifications. To obtain FRET values, regions containing focal adhesions at the cell periphery were high-pass filtered and focal adhesions structures in the different channels were aligned to correct for emission filter shifts. The FRET index (FI) was calculated for all pixels above threshold in both the CFP and the YFP channels by applying the equation below, pixel-by-pixel, to the image intensities recorded in the three channels. Threshold is set by the background level, and does not affect the ratio images where both denominator and numerator values are above it, but suppresses the undefined ratios otherwise. The fluorescence of CFP and YFP in the FRET channel was directly measured and subtracted from the total FRET values. The calculated net FRET was then normalized to donor fluorescence intensity (bleaching of CFP fluorescence was negligible):

$$FI = 100(\text{FRET} - \alpha\text{YFP} - \beta\text{CFP}) / \text{CFP},$$

where α and β are the measured 'bleed-through coefficients' of YFP and CFP fluorescence through the FRET channel. FI values were calculated based on three to ten consecutive time-lapse images and averaged for 11–32 cell samples. Ratio images of two components were calculated as previously described (Zamir et al., 1999). Briefly, images showing localization of CFP and YFP fusion proteins were high-pass filtered and the images were aligned. YFP/CFP ratio values were calculated for each pixel above threshold in both YFP and CFP channels. Ratio images were displayed using a spectral color look-up table ranging from 0.5 (blue) to 2 (red). To compensate for different expression levels and the different photon yields of CFP- and YFP-fusion proteins, the ratios were normalized by a constant that shifted their average values towards 1. For the presentation of ratio and FRET dynamics in Fig. 8, images were 'smoothed' by averaging 2×2 pixels in order to better visualize dynamic alterations avoiding fluctuating background noise. This was particularly useful for the high magnification used in the present study, where the pixel size (0.13 µm) is below the light microscope resolution.

Immunoprecipitation

NIH 3T3 cells were transfected with cDNA constructs as indicated, using LipofectaminePLUS. Cells were washed, 24 hours post transfection, with TBS (50 mM Tris-HCl, 150 mM NaCl) and lysed with NP40-lysing buffer (1% NP40, 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 2.5 mM EGTA 7.4, 1 mM NaF, 10 mM MgCl₂, 1 mM Na₃VO₄, and protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were centrifuged for 10 minutes at 15,000 g to remove insoluble material. The supernatant was used for immunoprecipitation using protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GFP antibodies (Roche, Mannheim, Germany), anti-p130CAS, or anti-FAK antibodies (both from Transduction Laboratories) for 2 hours at 4°C. The immunoprecipitates were separated by 10%

SDS-PAGE gel electrophoresis and transferred to nitrocellulose. Non-specific binding sites on the membrane were blocked using 5% BSA in TBS-T (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20); membrane was then probed for 1 hour at RT using anti-GFP (Roche), anti-PY (4G10) or anti-paxillin antibodies (both from Transduction Laboratories). After washing three times with TBS-T the membranes were incubated for 1 hour with horseradish-peroxidase-linked secondary antibodies and then developed using enhanced luminescence detection.

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