Calcium Rises Locally Trigger Focal Adhesion Disassembly and Enhance Residency of Focal Adhesion Kinase at Focal Adhesions*

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Focal adhesion kinase (FAK) activity and Ca2+ signaling led to a turnover of focal adhesions (FAs) required for cell spreading and migration. We used yellow Cameleon-2 (Ycam), a fluorescent protein-based Ca2+ sensor fused to FAK or to a FAK-related non-kinase domain, to measure simultaneously local Ca2+ variations at FA sites and FA dynamics. Discrete subcellular Ca2+ oscillations initiate both propagating and abortive Ca2+ waves in migrating U87 astrocytoma cells. Ca2+-dependent FA disassembly occurs when the Ca2+ wave reaches individual FAs, indicating that local but not global Ca2+ increases trigger FA disassembly. An unexpectedly rapid flux of FAK between cytosolic and FA compartments was revealed by fluorescence recovery after photobleaching studies. The FAK-Ycam recovery half-time (17 s) at FAs was slowed (to 29 s) by Ca2+ elevation. FAK-related non-kinase domain-Ycam had a faster, Ca2+-insensitive recovery half-time (11 s), which is consistent with the effect of Ca2+ on FAK-Ycam dynamics not being due to a general modification of the dynamics of FA components. Because FAK association at FAs was prolonged by Ca2+ and FAK autophosphorylation was correlated to intracellular Ca2+ levels, we propose that local Ca2+ elevations increase the residency of FAK at FAs, possibly by means of tyrosine phosphorylation of FAK, thereby leading to increased activation of its effectors involved in FA disassembly.

Cellular adhesion and migration involve remodeling and reorganization of focal adhesion (FA) sites. In FAs, aggregated integrins span the plasma membrane and mediate interactions between extracellular matrix components and cytoskeletal proteins. In migrating fibroblasts, FAs are mainly immobile, serving as traction points, except in restricted retraction cell edges, where they display centripetal movements (1). This local regulation of FA movement involves asymmetric signal transduction and probably directs cell polarization and migration. Despite the observation of an increasing Ca2+ gradient from front to rear in migrating fibroblasts (2, 3), Ca2+ is not often suggested to be a polarity signal in chemotaxis. This is surprising, given the large number of potential Ca2+-sensitive targets involved in integrin signaling and cell migration (4–8) and the tight spatio-temporal regulation of Ca2+ elevations (9). In support of a spatially restricted action of Ca2+ during cell migration, Ca2+ oscillations induce cell rear retraction in lymphocytes and keratinocytes (10, 11). Using fluorescent protein fusion constructs targeted to FAs, we showed recently (12) that Ca2+ elevations trigger local disassembly of FAs in human U87 astrocytoma cells. Here, we again made chimeric proteins using focal adhesion kinase (FAK) as an FA-targeting module and yellow Cameleon-2 (Ycam), a fluorescent protein-based Ca2+ sensor (13) to measure both local Ca2+ variations at FAs by fluorescence resonance energy transfer (FRET) half-time (15) mediated by the formation of integrin clusters. The central role of FAK in the formation of a functional FA is also emphasized by its scaffolding function, allowing direct interaction and translocation of signaling proteins such as Src, growth factor receptor binding protein-2, paxillin, phospholipase C-γ, phosphatidylinositol 3-kinase, and p130cas (16) toward FAs. Furthermore, FAK interacts with FA structural proteins, including integrins, talin, α-actinin and tensin, and via paxillin with both vinculin and F-actin (17). In FAK-deficient cells, reduced motility is accompanied by an increased number of FAs (18), suggesting that FAK tyrosine kinase activity is involved in the regulation of FA turnover (19). Expression of the enhanced yellow fluorescent protein; FRNK, FAK-related non-kinase domain; FRAP, fluorescence recovery after photobleaching; FCS, fetal calf serum; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; BAPTA, 1,2-bis(2-aminoethyl)ethylendiamine tetraacetic acid; FAT, focal adhesion targeting sequence; GFP, enhanced green fluorescent protein.
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Ca\(^{2+}\) rises triggered by FAK disassembly were investigated. These increases in FAK at FAs likely direct the activation of FAK at FAs. These considerations led to the examination of FAK-Ycam and FRNK-Ycam molecular dynamics using fluorescence recovery after photobleaching (FRAP). We report a rapid exchange of FAK-Ycam and FRNK-Ycam between cytosolic and FA compartments. FRNK-Ycam, which induced a decrease in tyrosine phosphorylation content at FA, had a shorter recovery half-time compared with that of FAK-Ycam. The local Ca\(^{2+}\) increase triggered FA disassembly prompted us to investigate the effect of Ca\(^{2+}\) on the dynamics of FAK-Ycam and FRNK-Ycam.

Expression of Plasmids and Transfection—As described previously (12), fluorescent FAK-targeted protein was made by fusion of FAK cDNA and 0.1 mg/ml gentamycin. A fluorescent FA-targeted protein was made by fusion of FAK cDNA and 0.1 mg/ml gentamycin.

**EXPERIMENTAL PROCEDURES**

Reagents and Cells—Eagle’s minimal essential medium, fetal calf serum (FCS), HEPES, t-glutamine, penicillin, streptomycin, gentamycin, and trypsin-EDTA solution were obtained from Invitrogen. Fura Red-AM, and bovine serum albumin (BSA) and incubated 30 min with PBS/3% BSA.

Cells were maintained at 37°C in a humidified incubator gassed with 5% CO\(_2\) in air on type I collagen-coated (0.06 mg/ml) plastic dishes in Eagle’s medium. Cells were isolated (JetStar Plasmid kit, Genomed) before transfectioning a similar protocol. All constructs were verified by sequencing. A fluorescent FA-targeted protein was made by fusion of FAK cDNA and 0.1 mg/ml gentamycin.

Expression of Plasmids and Transfection—As described previously (12), fluorescent FAK-targeted protein was made by fusion of FAK cDNA and 0.1 mg/ml gentamycin.

**RESULTS**

Local Active Ca\(^{2+}\) Oscillators Trigger Propagating Ca\(^{2+}\) Waves—Recently, we showed that Ca\(^{2+}\) spikes observed in migrating U87 cells represent a Ca\(^{2+}\) wave that spreads throughout the cell. Here, Ca\(^{2+}\) measurements restricted to FAs were made using FAK-Ycam. U87 cells having stable expression of FAK-Ycam were grown to sub-confluence on Matrigel and imaged in Ringer’s solution containing 10% FCS, conditions necessary to observe Ca\(^{2+}\) spikes (12) and functional integrins. We observed clear kinetic differences in Ca\(^{2+}\) elevations at discrete FAs (Fig. 1A), which is in agreement with a propagating wave. The delays measured for the appearance of Ca\(^{2+}\) waves varied among different FAs.
Ca\textsuperscript{2+} spikes at individual FAs located in different parts of a cell (Fig. 1C) allowed calculation of a Ca\textsuperscript{2+} wave speed of 20 ± 10 μm/s (n = 48 waves, 7 cells). An interesting feature is the presence of active Ca\textsuperscript{2+} oscillators, which reliably trigger Ca\textsuperscript{2+} wave propagation. These sites were defined as the nearby FAs displaying the earliest increase in Ca\textsuperscript{2+} during spiking (Fig. 1, compare red and light-blue FAs) and were characterized by a distinct and relatively constant frequency of oscillation. Between one and three active Ca\textsuperscript{2+} oscillators were detected in different cells (n = 22). Both regenerative, propagating Ca\textsuperscript{2+} waves (which couple to remote conveying Ca\textsuperscript{2+} oscillators; see Fig. 1B, first four spikes) and subcellularly restricted or abortive Ca\textsuperscript{2+} waves (which do not propagate throughout the cell; see Fig. 1B, spikes 5–7 of red FA) appear to be generated by active oscillators. Both types of waves probably share common underlying mechanisms, because the propagation speed for abortive Ca\textsuperscript{2+} waves (21 ± 12 μm/s; n = 52 waves, 9 cells) was the same as for regenerative waves. The location of active Ca\textsuperscript{2+} oscillators seems not to be correlated with the direction of cell migration, as we have observed polarized cells having active Ca\textsuperscript{2+} oscillators at both poles (not shown). However, it should be noted that our experiments were done without any directed signals (low density plating, homogeneous serum stimulation). Note also that our methods did not allow us to resolve whether FAs are the sites where integrin-dependent Ca\textsuperscript{2+} signaling is first generated. Nevertheless, we used FAK-Ycam, which concentrates FAs, to measure local Ca\textsuperscript{2+} rises around individual FAs in an effort to demonstrate the compartmentalized nature of Ca\textsuperscript{2+} elevations.

Local Subcellular Ca\textsuperscript{2+} Increases Trigger Disassembly of Neighboring FAs—We also reported that Ca\textsuperscript{2+} spikes trigger FA disassembly (12), based upon simultaneous measurements of global Ca\textsuperscript{2+} variations and dynamics of FAs or actin stress fibers. We tested whether the spatially restricted Ca\textsuperscript{2+} increases described above were capable of triggering adjacent FA disassembly (Fig. 2). Color overlays (blue, red, green) of three sequential images of EYFP emission (Fig. 2A) distinguish immobile FAs (black) versus motile FAs (rainbow). Disassembly of peripheral FAs (Fig. 2A, boxed areas 1 and 2), which corresponds to dissipation of EYFP fluorescence (Fig. 2, C and D, green traces) and subsequent cell-edge retraction (Fig. 2A, blue and red dominant colors in the zone close to areas 1 and 2), were coincident with global Ca\textsuperscript{2+} oscillations (calculated as the sum of subcellular Ca\textsuperscript{2+} increases; see Fig. 2B, red trace, spike 4). As described previously, this disassembly occurs simultaneously with retraction of associated stress fibers and cell-edge movement. The local Ca\textsuperscript{2+} rise in region 1 (Fig. 2B, green trace) mirrors the global Ca\textsuperscript{2+} signal, unlike for region 2 (Fig. 2B, blue trace). Disassembly of FA 2 (at 340 s; see Fig. 2D, green trace) is triggered by a local rise in Ca\textsuperscript{2+} in region 2 (Fig. 2B, blue trace; Fig. 2D, red trace). Although this early Ca\textsuperscript{2+} increase appears to be weakly propagated to region 1, disassembly of FA 1 occurs later at 370 s, coincident with a strong local Ca\textsuperscript{2+} increase in region 1 (Fig. 2B, green trace; Fig. 2C, red trace) which hardly propagated to region 2. These data demonstrate that disassembly of individual FAs is associated with local increases in Ca\textsuperscript{2+}. This spatial and temporal correlation was observed in all cells where Ca\textsuperscript{2+} increases in different parts of the cell were clearly separated in time, with the latency between the onset of local Ca\textsuperscript{2+} increases and FA disassembly being always shorter (15 ± 2 s, n = 7 FAs, 6 cells) when compared with global Ca\textsuperscript{2+} increases measured from the entire cell (39 ± 8 s, n = 7 FAs, 6 cells).

We reported previously (28) that integrin-dependent Ca\textsuperscript{2+} oscillations in U87 cells were reduced by depletion of Ca\textsuperscript{2+} stores and abolished by non-selective inhibition of Ca\textsuperscript{2+} channels by Cd\textsuperscript{2+} and La\textsuperscript{3+}. However, Ga\textsuperscript{3+}, which inhibits stretch-dependent Ca\textsuperscript{2+} channels, did not affect the generation of Ca\textsuperscript{2+} oscillations in U87 cells (not shown), unlike in keratinocytes (11). To show directly that Ca\textsuperscript{2+} increases because of Ca\textsuperscript{2+} entry cause FA disassembly, we bathed FAK-Ycam cells in Ca\textsuperscript{2+}-free external medium (with 10% FCS) containing 5 mM EGTA (to empty internal Ca\textsuperscript{2+} stores) and 5 μM ionomycin (Fig. 3). Upon local and repeated microperfusion of the same solution (but containing, in addition, 2 mM Ca\textsuperscript{2+}), transient increases in Ca\textsuperscript{2+} were observed (using Fura Red; see Fig. 3D, solid trace) to trigger FA disassembly (Fig. 3D, dotted traces). Note the retraction of the cell edge after Ca\textsuperscript{2+} application (Fig. 3B). Although we cannot rule out that the experimental protocol used might give rise to Ca\textsuperscript{2+}-sensitive outside-in integrin signaling, on balance, given the present knowledge concerning how external Ca\textsuperscript{2+} affects integrin function (for review, see Ref. 70), we feel it is quite plausible that the observed elevations in Ca\textsuperscript{2+} provoked by ionomycin-assisted Ca\textsuperscript{2+} entry trigger FA disas-
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**Fig. 2.** Spatio-temporal correlation of local Ca\(^{2+}\) increases and focal adhesion disassembly. Simultaneous measurements of subcellular changes in Ca\(^{2+}\) and FA dynamics in U87 cells using FAK-Ycam. A, color overlay of three sequential images (blue, red, and green at times i, ii, and iii in C and D) of EYFP emission (522 nm) in a FAK-Ycam cell having Ca\(^{2+}\) oscillations. Immobile FAs are black, and mobile FAs appear as rainbows (blue to green). Boxed areas 1 and 2 enclosing FAs 1 and 2 are shown enlarged (right panels; black arrows represent the direction of FA movement). Cell-edge retraction is visualized by the blue and red dominant colors in the zone close to areas 1 and 2. B, green and blue traces show local Ca\(^{2+}\) variations at FA 1 and FA 2, respectively (evaluated by changes in Y-cam FRET, R530/485); the red trace represents global Ca\(^{2+}\) oscillations measured from the entire cell surface. C and D, local Ca\(^{2+}\) variations (red traces) and FA dynamics (green traces, EYFP emission) at FA 1 (C) and FA 2 (D). Disassembly of FA 2 is associated with a Ca\(^{2+}\) rise in region 2 (dotted line at 340 s in B and D). The later disassembly of FA 1 is triggered by a Ca\(^{2+}\) increase in region 1 (dotted line at 370 s in B and C).

**Fig. 3.** Ionomycin-induced Ca\(^{2+}\) increases trigger FA disassembly. A–C, EYFP emission (522 nm) at FAs (boxed area is enlarged in C) in a FAK-Ycam cell before (A) and after (B) local microperfusion of 2 mM Ca\(^{2+}\) solution (bath contained 5 µM ionomycin and 0 µM Ca\(^{2+}\)/5 mM EGTA). Note the cell-edge retraction (B, arrow). D, decrease in Fura Red emission (585 nm, continuous trace) corresponds to increase in Ca\(^{2+}\) in the same cell. The onset of FA disassembly (dotted traces corresponding to FA 1 and FA 2 in C) is coincident with the rise in Ca\(^{2+}\).

**FAK Molecular Dynamics Are Characterized by a Fast Equilibrium between FAs and Cytosol**—In motile U87 cells, we also observed predominantly static FAs, as found in fibroblasts (11), together with restricted zones where FAs disassembled and formed, respectively, at the cell rear and at the migration front (12). FAs need to be relatively stable structures in time and space to support traction of the cytoskeleton necessary to propel the cell body (11). Cell-edge retraction triggered by FA disassembly stops when the next stable FA is encountered. We occasionally observed a slow redistribution of FAK-Ycam fluorescence from a disassembling FA to an adjacent FA (Fig. 4). As before, color overlays (blue, red, green) of three sequential images of EYFP emission (Fig. 4A) distinguish immobile FAs (black) from motile FAs (rainbow). This exchange of FAK (Fig. 4A, right panels), which occurs over several minutes, is seen as a loss of FAK-Ycam EYFP fluorescence from a disassembling FA 1 (Fig. 4A, dotted circle in upper right panel; Fig. 4B, green line) and an increase of EYFP fluorescence in an adjacent, (initially) stable FA 2 (Fig. 4A, upper right panel, solid circle; Fig. 4B, blue trace).

To test whether the disappearance of FAK-Ycam fluorescence during FA disassembly corresponds to the dissociation of a stable complex containing FAK and FA structural components, as suggested by Fig. 4, the molecular dynamics of FAK-Ycam exchange between the cytosolic and FA compartments were studied by FRAP (Fig. 5). Experiments were made to determine the turnover of FAK-Ycam at immobile FAs. The EYFP moiety of Ycam was bleached at individual FAs, and subsequent time-lapse imaging of bleached regions allowed visualization of FAK-Ycam movement during recovery (Fig. 5B, second column). The recovery of FAK-Ycam at FAs was surprisingly fast, with a half-time of 17 ± 4 s (Fig. 5D, n = 12 FAs), which, together with the 79 ± 4% recovery, indicates the presence of a small immobile fraction. This supports the idea that there is a large and rapid exchange flux of FAK between cytosolic and FA compartments, with FAK molecular dynamics being much faster compared with both FA formation and disassembly (which take >5 min). FRAP experiments done on small areas of the peripheral cytosol revealed a FAK-Ycam recovery half-time of 3 ± 1 s (Fig. 5C, n = 13), which is much faster than at FAs and is most likely diffusion-limited. This result indicates that additional factors, for example binding to various FA components, are involved in regulating FAK exchange between FAs and the cytosol. Therefore, the fast exchange of FAK between cytosolic and FA compartments contrasts with the stability of FAs and suggests that FAK forms transient interactions with FA components.

**FRNK Molecular Dynamics Are Faster than Those of FAK**—FRNK acts as a dominant-negative of FAK, lacking the N-
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**Fig. 4. Redistribution of FAK to adjacent FAs during FA disassembly.** A, color overlay of three sequential images (blue, red, and green at times i, ii, and iii in B) of EYFP emission (522 nm) in a FAK-Ycam-expressing cell showing mobile (rainbow) and static (black) FAs. Right panels, enlargements of the boxed area. Upper right panel, disassembly of an isolated FA (area enclosed by the dotted line); the other two encircled regions show FAK redistribution between FA 1 and the adjacent FA 2. Lower right panel, image subtraction at time iii minus time i; in this representation, loss of EYFP signal from the disassembling FA appears black, and gain of EYFP signal in the adjacent FA appears white. B, time course of EYFP signals for the two FAs encircled in A. Disassembly of FA 1 (red trace) is accompanied by a simultaneous increase in EYFP signal in FA 2 (blue trace, arrow in upper right panel in A) that was initially stable but then subsequently disassembled.

terminal domain, the kinase domain, and the Tyr\(^{397}\) autophosphorylation site, but including the C-terminal FA-targeting sequence (20). Accordingly, both the total amount of tyrosine phosphorylation (Fig. 6A) and FAK Tyr\(^{397}\) phosphorylation (Fig. 6B) were decreased in FRNK-Ycam-transfected cells compared with adjacent non-transfected cells. To test whether FAK and FRNK displayed the same molecular dynamics, FRAP experiments were done using FRNK-transfected cells. The recovery of bleached FRNK-Ycam at FAs (Fig. 5B, first row) was significantly faster than for FAK-Ycam (Fig. 5B, second row), with a recovery half-time of 11 ± 3 s (Fig. 5D, n = 13 FAs), whereas the immobile fraction was similar (16 ± 6%). However, no significant differences were noted between FAK-Ycam and FRNK-Ycam dynamics after cytosolic bleaching, with a FRNK recovery half-time of 2 ± 1 s (Fig. 5C, n = 8). These data indicate that the faster recovery of FRNK at FAs is not due to differences in cytosolic diffusion of FAK compared with FRNK, and that the slower recovery of FAK is due to a longer residency time at FAs.

**Calcium Elevation Prolongs FAK Association at FAs**—Only a few studies have reported a correlation between Ca\(^{2+}\) elevations and FAK-mediated increases in tyrosine phosphorylation (23–25). In U87 cells, thapsigargin-induced Ca\(^{2+}\) increases produce a rapid (<30 s) enhancement of FAK Tyr\(^{397}\) phosphorylation (12), as also seen for ionomycin-induced Ca\(^{2+}\) elevation (not shown). In agreement, Ca\(^{2+}\) buffering with BAPTA induced a dramatic reduction of FAK Tyr\(^{397}\) phosphorylation at FAs (Fig. 6C). Because enhanced migration associated with sustained FA turnover is dependent upon FAK activity (18, 19, 29), activation of FAK targets, and FA-localization of FAK (22, 30), it is possible that increases in Ca\(^{2+}\), which trigger FA disassembly, might also have regulatory effects on the activation and/or localization of FAK.

Therefore, we used ionomycin to artificially trigger Ca\(^{2+}\) elevation and then examined the molecular dynamics of FAK-Ycam and FRNK-Ycam in FA and cytosolic compartments by FRAP (Fig. 5). Despite extensive disassembly of FAs observed in most cells after ionomycin application (not shown), sufficient remaining stable FAs allowed us to carry out FRAP experiments. The FA residency time of FAK-Ycam was significantly increased (Fig. 5B, third row) after ionomycin treatment, with a recovery half-time of 29 ± 7 s, compared with 17 ± 4 s in control conditions (Fig. 5F, n = 18 FAs). Furthermore, the immobile FAK-Ycam fraction was slightly but significantly increased, to 32 ± 5%, compared with 21 ± 4% in control conditions. By contrast, the exchange of FRNK-Ycam between the cytosol and FAs was not affected by an increase of Ca\(^{2+}\) (recovery half-time = 11 ± 3 s, immobile = fraction 17 ± 9%; Fig. 5E, n = 10 FAs). Likewise, in the presence of ionomycin, the cytosolic recovery half-time of FAK-Ycam (3 ± 2 s; Fig. 5F, n = 13) was the same as in control (Fig. 5C). Thus, increases in Ca\(^{2+}\) slow FAK-Ycam dynamics at FAs, which are possibly linked to enhanced tyrosine phosphorylation, but not those of FRNK-Ycam, indicating that Ca\(^{2+}\) elevation does not result in a nonspecific modification of the dynamics of components at FAs. Furthermore, the FAK-Ycam recovery half-times (tens of seconds) are in sharp contrast with the much slower kinetics (>5 min) of FA disassembly (and formation), suggesting that a long-lived dissociation (or association) of FAK from (with) an FA complex occurs during these processes.

To verify that the Ca\(^{2+}\)-sensitivity of FAK-Ycam dynamics at FAs was unrelated to the conformational changes of Ycam induced by Ca\(^{2+}\) binding (13) after ionomycin treatment, we repeated FRAP experiments on cells transfected with FAK-GFP (34). As illustrated, both FAK-GFP and endogenous FAK were revealed in Western blots, with localization of FAK-GFP in FAs (Fig. 7A). Compared with FAK-Ycam dynamics in untreated cells (recovery half-time = 17 ± 4 s, immobile fraction = 21 ± 4%; see Fig. 5D), FRAP experiments showed that FAK-GFP dynamics at FAs were faster with recovery half-times of 10 ± 3 s (Fig. 7B, n = 34 FAs), with a similar immobile fraction of 24 ± 7%. These faster dynamics may be related to the smaller size of FAK-GFP compared with FAK-Ycam. After treatment with ionomycin, FAK-GFP recovery half-times (Fig. 7B) were significantly increased (22 ± 5 s; n = 26 FAs), with a small increase also in the immobile fraction (34 ± 6%). These slowing effects of increased intracellular Ca\(^{2+}\) on FAK-GFP dynamics are similar to those found for FAK-Ycam after ionomycin treatment (recovery half-time = 29 ± 7 s, immobile fraction = 32 ± 5%; see Fig. 5).

**DISCUSSION**

Despite the relative immobility of most FAs and their life span on the order of at least several minutes, this study shows that the association/dissociation of FAK and FRNK with FAs is a fast dynamic process, with increases in Ca\(^{2+}\) leading to longer association of FAK but not FRNK at FAs. This result is the first indication that FAK signaling might be regulated by modification of its partitioning between FAs and the cytosol. Note that both FA formation and disassembly have much slower kinetics, and that the molecular dynamics of FAK are about 10-fold faster compared with other FA proteins (31–33). Simultaneous measurements of local Ca\(^{2+}\) variations at FAs and FA dynamics reveal that Ca\(^{2+}\)-dependent FA disassembly occurred when the Ca\(^{2+}\) wave, which is composed of transient Ca\(^{2+}\) subcellu-
Fig. 5. Regulation of the molecular dynamics of FAK-Ycam at FAs by Ca\textsuperscript{2+} elevation. A, 10βT7 cells expressing FRNK-Ycam (paired images, 1), FAK-Ycam in basal Ca\textsuperscript{2+} condition (paired images, 2), or after ionomycin-induced Ca\textsuperscript{2+} elevation (paired images, 3) imaged before and after recovery from bleaching (respectively left and right panels) of an isolated FA (boxed areas). B, time-lapse sequences showing recovery after bleaching of corresponding FAs in A (bp, before photobleaching; ap, after photobleaching; the time after photobleaching is indicated in s; far, fluorescence after recovery). The rows correspond to sequential images of the boxed FAs in the FRNK-Ycam cell (1), the FAK-Ycam cell (2) and the FAK-Ycam cell + ionomycin (3) in A. C–F, kinetics of recovery of FAK-Ycam and FRNK-Ycam fluorescence in FA and cytosolic compartments after bleach. Fluorescence intensity in the bleached region was measured and expressed as relative recovery. Note the shorter recovery half-time at FAs for FAK-Ycam compared to FRNK-Ycam (D) and the longer recovery half-time for FAK-Ycam after ionomycin (F). Data are mean ± S.D. (representative error bar at the end of each trace). The sizes of peripheral FAs in FRAP experiments were always between 2–3 μm diameter.

lar elevations, reached the vicinity of the FA. Because compartmentalized Ca\textsuperscript{2+} oscillations trigger local FA disassembly and Ca\textsuperscript{2+} elevation or buffering induce, respectively, an increase or decrease in FAK autophosphorylation, we propose that Ca\textsuperscript{2+}-induced increases in FAK association with FAs might be involved in FA disassembly.

FAK functions as a molecular adaptor interacting with both signaling and structural proteins (16), which is consistent with FAK being a multifunctional protein playing both signaling and structural roles (17). The continuous fast exchange of FAK-Ycam between cytosolic and FA compartments reported here signifies a signaling function for FAK, given the immobility of most FAs in migrating cells (1, 19). In control conditions, after photobleaching FAK-Ycam, we found an unexpectedly fast recovery half-time of 17 s, which is the fastest so far described for an FA protein. This finding is consistent with recent work showing that FAK autophosphorylation is intermolecular, and that FAK cannot form stable homo-oligomers (15); rather, FAK requires external proteins for transient transphosphorylation and subsequent action on its targets. By comparison, recovery half-times after photobleaching of 2–5 min (31) or >10 min (32) have been reported for the integrin β3 subunit; for α-actinin, the recovery half-time after photobleaching was 2–3 min (32, 33). Even if β3 integrins move slowly inwards at FAs (31), our results indicate that FAK and FRNK rapidly associate and dissociate from a more stable platform at FAs composed of at least integrins. In agreement with a predominant signaling role for FAK, chromophore-assisted laser inactivation of FAK at FAs does not lead to stress fiber detachment (34). Nevertheless, redundant interactions that characterize FA-associated proteins could underlie protein exchange without dissipation of FA architecture and might reconcile fast FAK dynamics with a structural function. Consistently, low affinity interactions between FA components and integrins have been described (35), which may facilitate protein-exchange dynamics. Note that chromophore-assisted laser inactivation of α-actinin, which exchanges between FAs and the cytosol more slowly than FAK, leads to disruption of integrin-cytoskeleton interactions (34). The fast dynamics of FAK-Ycam suggest that FAK exchange between FA and cytosolic compartments may be constant during the relatively slower process of FA disassembly (>5 min). Thus, FAK-Ycam fluorescence probably reflects the size of the integrin-containing platform with which FAK associates and which is more stable.

The identical ability of FAK and FRNK to localize at FAs, which are unaltered by the Ycam tag, is due to the FAT sequence (36) that contains binding sites for paxillin and talin (37, 38), both of which seem to be involved in FA targeting. The shorter FA association time for FRNK compared with FAK may result from FRNK being unable to mimic FAK signaling or from a difference in affinity for FA components. Indeed, in FRNK, an integrin interaction site in the FAK N-terminal domain is absent (39), along with the kinase domain and the Tyr\textsuperscript{397} autophosphorylation site responsible for recruitment and activation of Src-kinase (40).

In agreement with the predominant signaling hypothesis for FAK, the region between FAT and the kinase domain, as well as the N-terminal domain, are not necessary for adhesion-dependent FAK tyrosine phosphorylation and FAK-dependent paxillin tyrosine phosphorylation, and thus are probably not involved in FAK targeting to integrins and downstream signaling mediated by FAK (41). However, the FAT domain is necessary for FAK and FRNK to promote adhesion-dependent FAK tyrosine phosphorylation and for the dominant-negative function of FRNK (30, 41). Thus, it seems that regions underlying binding capabilities are mainly present in the FAT domain,
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Thus supporting the idea that events linked to FAK tyrosine phosphorylation activity determine kinetics of FAK and FRNK residency at FAs. Both FAT and Tyr\(^{397}\) are required for complete tyrosine phosphorylation of downstream FAK targets like paxillin (20, 21, 41). Formation of Src-kinase/FAK complexes allows subsequent trans/cis-phosphorylation of other FAK tyrosine residues, inducing maximal FAK tyrosine kinase activity (42) and direct or indirect recruitment of FA components (16). Like others (43), we found that transfection of FRNK leads to reduced phosphorylation of endogenous FAK at FAs, as well as reduced global FA tyrosine phosphorylation, probably because of decreased tyrosine phosphorylation of paxillin, p130cas, and tensin (20, 21, 44). Therefore, the faster recovery half-time of FRNK-Ycam at FAs may result from either a reduction in direct docking of FRNK at FA-localized proteins interacting with Tyr\(^{397}\) or a decreased amount of FA-phosphotyrosines and, hence, a reduced number of potential interaction partners (43, 45).

In support of intrinsic differences in FA docking, a C-terminal domain mutation which disrupts paxillin binding to FAK and FRNK affects only FA localization of FRNK but not FAK targeting (22, 30, 37). On the other hand, even if the N-terminal domain is unnecessary for complete integrin-dependent kinase activity of FAK (46) or FAK-dependent tyrosine phosphorylation of paxillin (41), its interaction with integrin (39) may act to stabilize and extend FAK association at FAs. Indeed, the N-terminal domain of FAK has been demonstrated to be involved in the control of FAK activity (15, 47, 48).

Several reports demonstrate that migration speed is increased by Ca\(^{2+}\) signaling, for example in astrocytoma (28), smooth muscle cells (49), neutrophils (10), and neurons (50). Here, we have directly linked local Ca\(^{2+}\) variations to the migration process by showing that Ca\(^{2+}\)-dependent FA disassembly is associated with temporally and spatially restricted Ca\(^{2+}\) increases around individual disassembling FAs. The migration process is complex, involving the regulation of cell protrusion and retraction both in space and time (51), characteristics shared by the complex patterns of Ca\(^{2+}\) signaling reported here (Fig. 1). In cerebellar granule cells, Ca\(^{2+}\)-dependent migration is correlated with the amplitude and frequency of Ca\(^{2+}\) spikes (50), which may regulate different steps during migration. Migrating fibroblasts in the late stages of wound healing exhibit an increasing gradient of free Ca\(^{2+}\) from the front to the rear (2, 3), supporting a role for spatially restricted Ca\(^{2+}\) variations in the disassembly of FAs. Indeed, this might represent a mechanism by which the cell controls its polarity during migration. Oscillatory Ca\(^{2+}\) signaling may serve to prevent inappropriate FA disassembly, which may occur during sustained Ca\(^{2+}\) elevation, given the massive FA disruption seen for ionomycin-challenged cells.

It is widely held that the frequency and amplitude of Ca\(^{2+}\) signals allow activation of specific Ca\(^{2+}\) targets (54–57). High Ca\(^{2+}\) triggers diffusion of integrins out of FAs in adherent fibroblasts (52). Consistently, Ca\(^{2+}\) increases lead to calpain activation and the liberation of a fraction of \(\beta_2\) integrins, which are tethered to cytoskeletal components (53). The \(\beta_2\) targets involved in FA disassembly might be more sensitive to high levels of Ca\(^{2+}\), because only strong local Ca\(^{2+}\) elevations appear effective in triggering FA disassembly (Fig. 2). However, further studies are needed to explain why all FAs do not disassemble in response to massive ionomycin-induced Ca\(^{2+}\) increases, and to explain why FAs respond to a given Ca\(^{2+}\) spike and not the previous ones. One possibility is that repetitive increases in Ca\(^{2+}\) around individual FAs are necessary to raise permissive signals beyond a putative threshold, thereby triggering disassembly, as has been proposed previously (58) for repetitive targeting of microtubules to individual FAs (which also promotes disassembly). In any case, the data presented here do not permit us to identify precisely the molecular processes underlying the generation of Ca\(^{2+}\) oscillations nor to conclude that FAs are the exclusive sites of the active Ca\(^{2+}\) oscillators which initiate Ca\(^{2+}\) wave propagation. However, in cultured astrocytes, endoplasmic reticulum-associated proteins...
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(calreticulin and type 2 IP\textsubscript{3} receptors) and mitochondria have been described as Ca\textsuperscript{2+} wave-amplification sites (69).

Currently, the precise Ca\textsuperscript{2+} targets underlying FA disassembly are unknown, but they potentially include calciun (5), myosin light chain kinase (8), calpain (6, 33), calreticulin (7, 58–60), and the FAK-related proline-rich tyrosine kinase 2 (Pyk2) (61–63). Ca\textsuperscript{2+}-dependent changes in FAK tyrosine phosphorylation activity have been described (23–25), and we found that Ca\textsuperscript{2+} elevation rapidly (<1 min) increases FAK Tyr\textsuperscript{397} phosphorylation (12), whereas Ca\textsuperscript{2+} buffering with BAPTA reduces FAK Tyr\textsuperscript{397} phosphorylation at FAs. Because FAK localization to FAs and autocatalytic activation (15, 40) are involved in the subsequent Src activation and recruitment that enhances FAK activity (42), and both FAK (18, 19) and Src (64, 65) activity are linked to FA turnover, FAK may well be one of the Ca\textsuperscript{2+} targets involved in Ca\textsuperscript{2+}-dependent FA disassembly. In support of this idea, our FRAP experiments show that when [Ca\textsuperscript{2+}] is high, the FA-associated state of FAK-Ycam at FAs and in FAK-deficient fibroblasts (67) does not involve differences in signaling activation and increases in FA turnover and migration speed (12). We directly verified that Ca\textsuperscript{2+}-induced Src activity are linked to FA turnover, FAK may well be capable of rapidly regulating FA stability during cell spreading and migration, in response to both local (FA) and remote (cytosolic) signals. Indeed, the fast flux of FAK between FA and cytosolic compartments raises the possibility that FA-associated FAK may be replaced by FAK having modified activity coming from the cytosol. Finally, our results indicate that regulation of FAK signaling might be achieved by changes in its association time with FA partner components.

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