

Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells

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Summary

Cellular locomotion is driven by repeated cycles of protrusion of the leading edge, formation of new matrix adhesions and retraction of the trailing edge. In this study we addressed the molecular composition and dynamics of focal complexes, formed under the leading lamellae of motile cells, and their maturation into focal adhesions. We combined phase-contrast and fluorescence microscopy approaches to monitor the incorporation of phosphotyrosine and nine different focal adhesion proteins into focal complexes in endothelial cells, migrating into an *in vitro* 'wound'. We show that newly formed complexes are located posterior to an actin-, VASP- and α -actinin-rich region in the lamellipodium. They are highly tyrosine phosphorylated, contain β 3-integrin, talin, paxillin and low levels of vinculin and FAK, but are apparently devoid of zyxin and tensin. The

recruitment of these proteins into focal complexes occurs sequentially, so that their specific protein composition depends on their age. Interestingly, double color, time-lapse movies visualizing both paxillin and zyxin, indicated that the transition from paxillin-rich focal complexes to definitive, zyxin-containing focal adhesions, takes place only after the leading edge stops advancing or retracts. These observations illuminate, for the first time, early stages in focal complex assembly and the dynamic process associated with its transformation into focal adhesion.

Supplemental data available online

Key words: Cell motility, Focal adhesions, Focal complexes, Lamellipodia, Endothelial cells, Zyxin

Introduction

Cell adhesion to the extracellular matrix (ECM) plays multiple roles in the regulation of cell behavior and fate. In most adhesion sites, integrin receptors mediate the binding to the ECM via their extracellular domains, and interact via their cytoplasmic moieties with the actin cytoskeleton (Hynes, 1992; Martin et al., 2002). The intracellular domain of integrin-mediated adhesions contains a large number of proteins, some of which directly mediate or strengthen the mechanical linkage between the ECM and the cytoskeleton, while others participate in adhesion-mediated signaling (reviewed by Critchley, 2000; Geiger et al., 2001; Petit and Thiery, 2000). Recent studies have shown that integrin-mediated adhesions are not all alike and different molecular and cellular variants may be distinguished (see Zamir and Geiger, 2001). 'Classical' focal adhesions (FA) are usually located at the cell periphery, are highly tyrosine phosphorylated, and contain such proteins as α v β 3 integrin, vinculin and paxillin. At more central positions, 'fibrillar adhesions' are found in association with fibronectin fibrils, containing α 5 β 1 integrin, tensin and little or no phosphotyrosine (Zamir et al., 1999). At the cell periphery, mainly along the leading lamella of motile cells, small matrix adhesions, denoted 'focal complexes' (FX) are formed (Nobes and Hall, 1995). FX are short-lived structures, containing β 3-integrin (Ballestrem et al., 2001), vinculin (Rottner et al., 1999b), paxillin, α -actinin (Laukaitis et al., 2001) and possibly Arp2/3 (DeMali et al., 2002). It has been established that the three forms of ECM adhesions described above represent different stages in the interaction of cells with the matrix; thus,

FX are early adhesions, which transform into FA following the activation of Rho-A (Ballestrem et al., 2001; Rottner et al., 1999b) or as a result of external mechanical perturbation (Galbraith et al., 2002; Riveline et al., 2001). Fibrillar adhesions, in contrast, arise from FA following actomyosin-dependent centripetal displacement of ECM-associated fibronectin receptors (Pankov et al., 2000; Zamir et al., 2000).

In motile cells, matrix adhesion plays a central and direct role in the motile process. Polar protrusions at the leading edge produce many FX, some of which develop into stable FA. FA, in turn, play a dual role in motility; they provide robust anchors to the ECM, allowing the contractile actomyosin system to pull the cell body and trailing edge forward (Lauffenburger and Horwitz, 1996; Small et al., 1996) and they may also restrain the migration process (Huttenlocher et al., 1996).

In order to characterize the early stages in FA development, we have compared the protein composition of FX and bona fide FA. We show here that FX accumulate proteins during the protrusion of the lamellipodium, in a hierarchical manner, reaching a protein composition that is distinct from that of FA. Most notably, FX do not contain zyxin. Furthermore, our observations indicate that the transformation of FX into zyxin-containing FA depends on local retraction of the leading edge.

Materials and Methods

Cell culture and formation of 'in vitro wound'

Porcine aortic endothelial (PAE) cells (obtained from Avrum Gotlieb, Toronto general Hospital, Canada) were cultured in medium 199 with

Earle's salts, L-glutamine and NaHCO₃ (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% FCS and antibiotics (Biological Industries, Beit Haemek, Israel), at 37°C in a 5% CO₂ humidified incubator. For 'wound' experiments 4×10⁵ cells from early passages (5-15) were seeded on an 18 mm cover glass in a 35 mm plate, forming a confluent monolayer within 12 hours. An ~500 μm wide scratch was introduced into the monolayer using a plastic micro-tip, 24-36 hours after seeding.

To inhibit cellular contractility, wounded cells were incubated either with 30 μM Y27632, a Rho-kinase inhibitor (Calbiochem, San Diego, CA, USA) or with 100 μM 1-(5-iso-quinolinesulfonyl)-2-methylpiperazine (H-7; I-7016; Sigma Chemical Co., St Louis, MO, USA) for 30 minutes.

cDNA constructs and time-lapse microscopy

Cloning of GFP-β₃-integrin and GFP-paxillin has been described previously (Ballestrem et al., 2001; Zamir et al., 1999). Paxillin was re-cloned into pEYFP-C3 (Clontech, Palo-Alto, CA, USA) using *HindIII* and *XbaI*. CFP-zyxin was obtained by excising zyxin from GFP-zyxin (a gift from Jurgen Wehland, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) with *EcoRI* and *BamHI* and cloning it into pECFP-N1 (Clontech, Palo-Alto, CA, USA). Constitutive active (L61) Rac in pRK5 (provided by Kurt Ballmer-Hofer, Paul Scherrer Institute, Villigen, Switzerland) was co-transfected with pECFP to allow identification of transfected cells. Transient transfections of PAE cells were done by electroporation using a GENE PULSER II (Bio-Rad, Hercules, CA, USA). Cells were seeded on glass bottomed plates coated with fibronectin (5 μg/ml) and experiments were carried out 24-36 hours later. Time-lapse movies of the fluorescently tagged cells were usually acquired with a X100/1.4 objective at 1-minute intervals. Phase-contrast time-lapse movies were acquired with a ×10/0.25 objective at 30-second intervals. Cells were maintained on the microscope stage, in F12 medium (Biological Industries, Beit Haemek, Israel) + 30 mM Hepes supplemented with 10% FCS in a 37°C heated chamber.

Immunofluorescence staining

Cells were fixed-permeabilized as described previously (Ballestrem et al., 2001; Zamir et al., 1999) with the addition that the first step was done on the microscope stage.

The primary antibodies used in this study included: rabbit anti-phosphotyrosine (PT40; kindly provided by Israel Pecht and Arie Licht, The Weizmann Institute), mAb anti-phosphotyrosine (4G10; Upstate Biotechnology, Charlottesville, VA, USA), mAb anti-paxillin, anti-tensin and anti-FAK (Transduction Laboratories, Lexington, KY, USA), mAb anti-vinculin (clone hVin-1), mAb anti-α-actinin, mAb anti-talin (clone 8d4; Sigma Chemical Co., St Louis, MO, USA), mAb anti-zyxin (gift from Daniel Louvard, Institut Curie, Paris, France), mAb anti-VASP (gift from Jurgen Wehland, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany), rabbit anti-zyxin (B71, kindly provided by Laura Hoffman and Mary Beckerle, Huntsman Cancer Institute and Department of Biology, University of Utah, Salt Lake City, Utah, USA).

Secondary antibodies were all from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Digital microscopy

Phase-contrast and immunofluorescent images were taken with the DeltaVision system (Applied Precision Inc., Issaquah, WA, USA) on an Axiovert 125 TV inverted microscope (Zeiss, Oberkochen, Germany). Image acquisition and processing were controlled by a Silicon Graphics workstation model O₂ (Mountain View, CA, USA) using the Resolve3D and Priism software packages (Applied Precision Inc., Issaquah, WA, USA).

Phase-contrast and immunofluorescent image alignment

Phase-contrast movies were acquired with a ×10/0.25 air objective to visualize a large field of cells. Following fluorescence labeling, images corresponding to these fields were acquired with ×100/1.4 or ×63/1.4 oil objectives. Alignment of the phase-contrast movie with the fluorescent images was carried out by marking 20 recognizable points in both the last phase-contrast exposure and the corresponding fluorescent image. Then, the XY-shift was estimated from the difference in the center of mass of the two sets of points, the magnification from the ratio of the average distances between all points in each set and their center of mass, followed by Least Square Fitting of the two-dimensional matrix, adjusting the phase-contrast image points to those in the fluorescent image.

Fluorescent image analysis

Fluorescent images were high pass filtered (by subtracting from each pixel the average intensity in a 20×20 pixel box around it). Filtered images were used to produce pixel-by-pixel ratios as previously described (Zamir et al., 1999). In order to quantify features in various regions along the cell's front, 12-19 cells that were protrusive at the time of fixation were tested. For each cell two polygons were drawn on the fluorescent images: one surrounding FX in the leading edge and the second enclosing definitive FA in a more central region of the cell. We used phosphotyrosine (PY) fluorescence intensity above a threshold to create a segmentation mask defining adhesion sites (FX or FA). A second mask, created by a threshold in the fluorescent intensity of a labeled FA protein, defined the positively stained loci of this protein.

The degree of overlap of a particular FA protein with FX or FA, (which we refer to as 'percent co-localization'), was quantified by dividing the total fluorescence intensity of PY in all pixels shared by both masks (the 'overlap mask'), by the total fluorescence intensity of PY in the PY mask. Summing intensities within positively stained masks is insensitive to background pixels or to the exact threshold levels, and yields robust quantitative evaluation for co-localization of two labels.

To compare the fluorescence intensity of a protein in FX to FA we used the average intensity in the overlap mask area within the polygons.

Statistical analysis of distributions, correlation and linear regression was carried out using the JMP IN software, version 4.0.3 (SAS Institute Inc., Cary, NC, USA).

Images were arranged for publication using Adobe PhotoShop v5.0.2ME (Adobe Systems Inc., San Jose, CA, USA).

Results

Formation of FX and FA during protrusion and retraction of the leading edge

Within minutes after 'wounding' of a confluent endothelial monolayer, spreading of the cells was apparent, followed by their migration into the wound. Time-lapse microscopy revealed that the motion of the lamellipodium consists of protrusion and retraction cycles, with a typical duration of 3-10 minutes. The speed of the protrusion was variable, ranging from 0.2 to 2.5 μm/minute, and the average speed of migration of the cells as a whole was about 0.5 μm/minute. To visualize the formation of FX the cells were transfected with GFP (green fluorescent protein)-β₃-integrin [characterized by Ballestrem et al. (Ballestrem et al., 2001)] and the organization of small fluorescent adhesion sites along the cell's front was monitored. Examination of these time-lapse movies revealed that during advancement of the lamellipodium FX form continuously under the leading edge. They remain stationary, relative to the

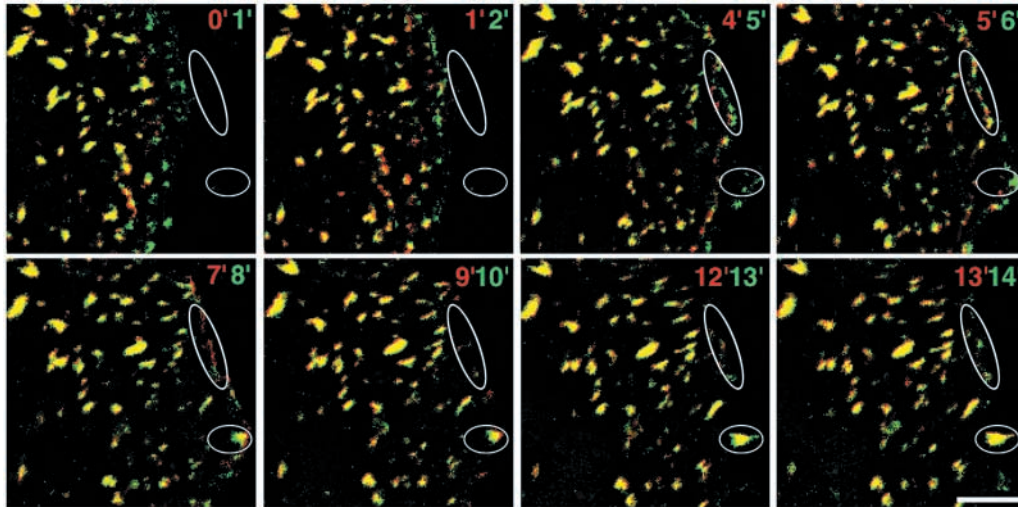


Fig. 1. Formation of focal complexes (FX) and their development into focal adhesions (FA). Porcine aortic endothelial cells were transfected with GFP- β_3 -integrin and cultured to confluence. The monolayer was scratched, creating a 'wound' and time lapse movies of transfected cells, migrating into the 'wound', were made at 1-minute intervals between exposures (see also Movie 1: <http://jcs.biologists.org/supplemental/>). For easier visualization of the dynamic process captured in this movie we superimposed sequential 'current' and 'current + 1 minute' images and colored them red and green, respectively. Structures appearing yellow remained unchanged between the two time points; structures in green are new, and structures in red disappeared before the second exposure. Notice that until 7 minutes there is active protrusion, followed by retraction (until 13 minutes), and the sequence ends with the beginning of a new protrusion. During the protrusive phase every minute new FX are formed, while 1- to 2-minute-old complexes tend to fade and disappear. Upon retraction, most of the FX disappear (in solid line oval) while a few FX grow into FA (dashed line oval). The growing FA expands towards the cell center. Scale bar: 5 μ m.

substratum, for 1-2 minutes, and then dissolve as the lamellipodium moves forward (see area marked by the solid line oval in Fig. 1). During retraction of the lamella, most of these FX disappear, and the remaining few elongate centripetally and transform into typical FA (dashed line oval in Fig. 1). To visualize the changes in FX and FA formation and distribution, we present here two-color images, showing the location of GFP- β_3 -integrin-containing adhesion sites at two time points, 1-minute apart (see also Movie 1: <http://jcs.biologists.org/supplemental/>). We have also transfected cells with GFP-paxillin and observed essentially the same dynamics (data not shown). Fixation of the migrating cells 'captures' some regions during protrusion and others during retraction. Immunolabeling of these cells for F-actin and paxillin reveals that protruding regions contain arrays of FX embedded in a diffuse meshwork of actin (solid line oval in Fig. 2A), while retracting regions of the lamella are outlined by large FA connected to stress fibers (broken line circle, Fig. 2A).

The molecular composition of FX differs from that of FA

To determine the molecular properties of FX we have monitored the dynamics of the leading edge of migrating endothelial cells by phase-contrast microscopy, and labeled the cells for different FA proteins (Fig. 2B). Labeling of GFP- β_3 -integrin-transfected cells for phosphotyrosine (PY) showed nearly complete co-localization of the two labels in both FX (Fig. 2C, solid line oval) and FA (Fig. 2C, broken line circle) at the cell periphery, indicating that PY in the leading edge area is an excellent marker of newly formed and mature adhesions.

Double labeling of migrating cells for PY and nine different FA-associated proteins was carried out and correlated with the

protrusive activity of the lamellipodium prior to fixation of the cells. Comparison of labeling patterns obtained with antibodies to the different FA proteins indicated that all the proteins except tensin (which is mainly associated with fibrillar adhesions; see supplementary Fig. S1D: <http://jcs.biologists.org/supplemental/>), co-localize with PY in FA. However, their association with FX was considerably more diverse. VASP and α -actinin were abundant along a thin band at the anterior margins of the leading edge, located 1-2 μ m in front of, and to a lesser extent, between the PY-rich FX. β_3 -Integrin, talin, paxillin, vinculin and FAK co-localized with PY in FX to a variable extent, while zyxin and tensin were consistently absent from these early adhesion sites. Representative examples for each of these protein groups are shown in Fig. 3 (for the full repertoire see supplementary Fig. S1: <http://jcs.biologists.org/supplemental/>).

Next, we set out to quantify the relative abundance of the different proteins in FX and FA. To this end we selected protruding regions along the lamellipodium, containing small dot-like structures (FX) close to the cell edge, and regions farther from the cell edge, containing large elongated FA. For each region we determined the extent of localization of the different proteins relative to PY (detailed in Materials and Methods). 12-19 cells were used for the analysis of each protein. All the FA proteins tested (i.e., β_3 -integrin, talin, paxillin, vinculin, FAK, VASP, α -actinin, and zyxin), except tensin, showed very high degrees of co-localization with PY at definitive FA, in the range of 80-90% (Fig. 4A). Localization of tensin in definitive FA was lower and variable, as the protein was primarily associated with fibrillar adhesions as previously described (Zamir et al., 1999). Quantification of co-localization of the various FA proteins in FX indicated that β_3 -integrin had the highest percentage co-localization with PY (>80% \pm 5%).

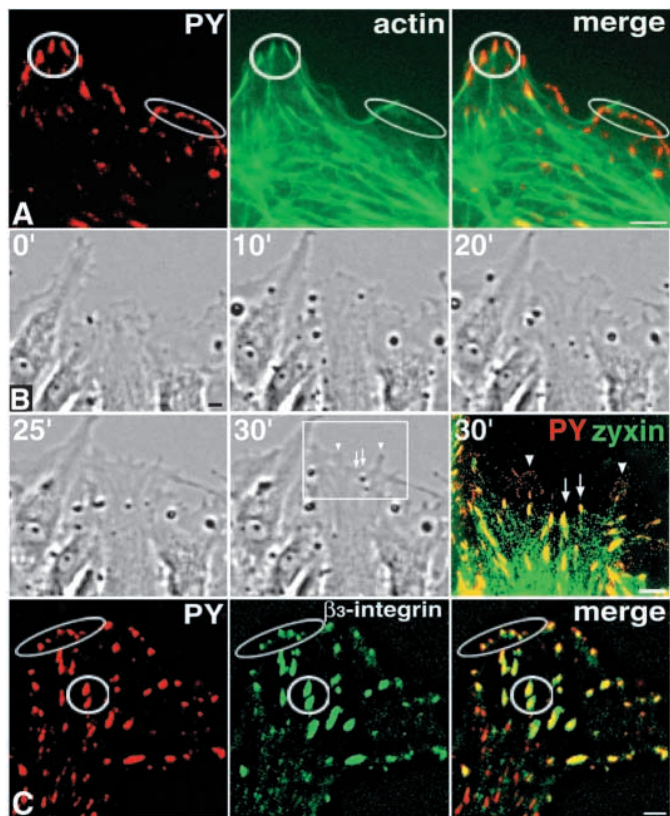


Fig. 2. Spatial relationships between paxillin, actin, phosphotyrosine (PY), zyxin and β_3 -integrin in FA and FX. Endothelial cells migrating into an in-vitro 'wound' were fixed and stained. (A) In a cell labeled for paxillin and actin we can distinguish a region that was protruding at the time of fixation (marked by a solid line oval) next to a region that was retracting (marked by a broken line circle). At the base of the protruding lamellipodium a rim of small FX can be seen surrounded by actin meshwork. At the edge of the retracting region large elongated FA extend towards the cell center, connected to stress fibers. (B) Leading edge movements were monitored by time-lapse microscopy prior to fixation and correlated with the pattern of protein distribution. For example here, a cell stained for PY and zyxin is shown along with frames from its corresponding phase-contrast movie. Retracting regions (marked by arrows) contain zyxin-positive FA, while protruding regions (marked by arrowheads) are lined by zyxin-negative FX. (C) A cell expressing GFP- β_3 -integrin was labeled for PY. Co-localization of the two labels is nearly complete both in FX (marked by the solid line oval) and in FA (marked by broken line circle) near the leading edge. In more central regions of the cell (not relevant for this work) $\alpha_5\beta_1$ -integrins replace $\alpha_v\beta_3$ -integrins. Scale bar: phase-contrast images, 10 μ m; others, 5 μ m.

Talin and paxillin were next with values ranging from 20% to 80% and means of 60% and 50%, respectively. Vinculin and FAK showed lower levels of co-localization with PY with average values of 30% and 25%, respectively. VASP and α -actinin were co-localized with PY to even lower levels of about 20%, while tensin and zyxin were hardly detectable in these sites (Fig. 4B). Comparison of the average labeling intensity of the tested components in FA and in FX showed that the intensity of PY, β_3 -integrin and talin is one and a half times higher in FA compared to FX, while the labeling for paxillin,

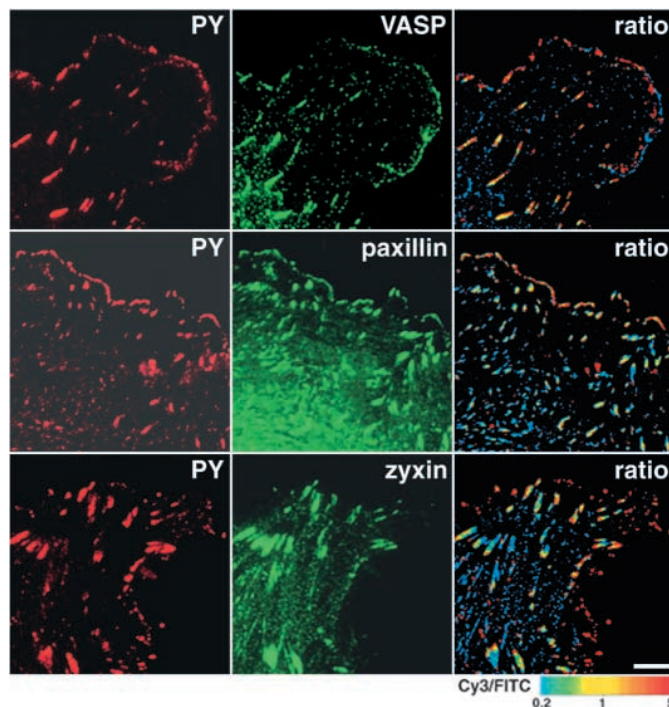


Fig. 3. Differential distribution of FA proteins in FX and FA. Endothelial cells migrating into an in-vitro 'wound' were fixed and immunolabeled for PY (left column) and different FA proteins (middle column) as indicated. Analysis of the differential distribution of the different proteins relative to PY was assisted by calculation of pixel-to-pixel ratio images (right column). VASP forms a chain of 1-2 μ m dots, located in front of the PY-positive FX while it co-localizes with PY in FA. Paxillin localizes extensively in both FA and FX. Zyxin appears both in a wavy pattern along actin and in FA; however, it is completely absent from FX at the leading edge. Scale bar: 5 μ m.

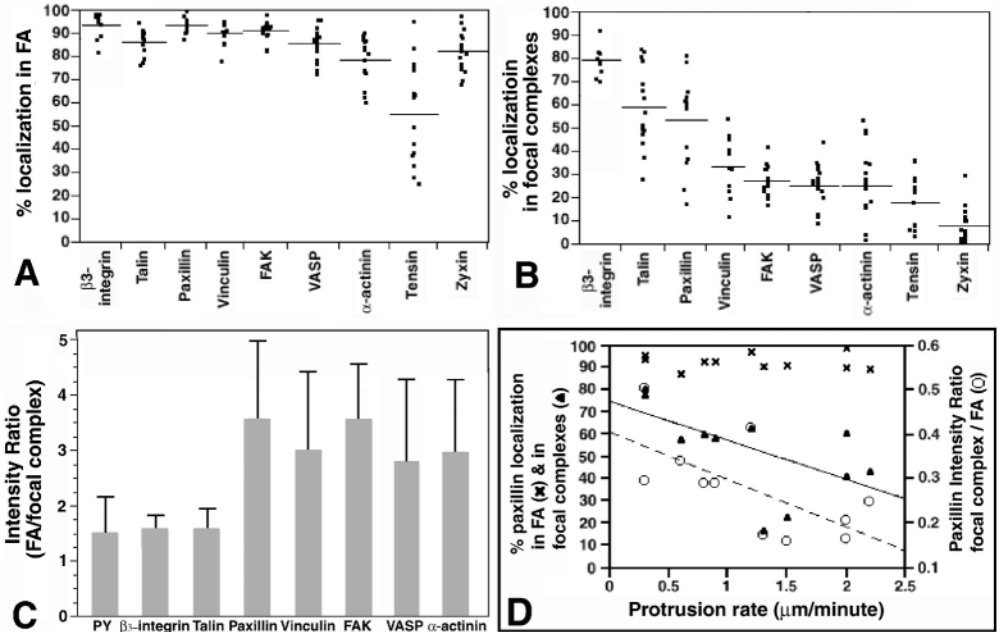
vinculin, FAK, VASP, and α -actinin is, on the average, three times more intense in FA relative to FX (Fig. 4C).

Molecular consolidation of FX is a progressive process that depends on the rate of lamellipodial protrusion

A striking feature of newly formed FX is the wide variability in their labeling for the different FA proteins. Interestingly, we noticed, that the variability in the molecular composition of individual complexes was 'regional'. Thus, FX within the same area of the lamellipodium were usually considerably more uniform. This observation raised the possibility that the local dynamic properties of the leading edge might affect FX composition.

To directly test this hypothesis we have examined the relationships between FX composition and the rate of protrusion of the leading edge in the same region, as visualized by phase-contrast time-lapse movies. We have specifically applied this analysis to one of the most prominent FX (and FA) proteins, namely paxillin. As shown in Fig. 4D, localization of this protein in FA was not affected by the rate of protrusion of the nearby lamella. However, paxillin association with FX was inversely correlated ($r=-0.6$, $P<0.05$) with the rate of local protrusion (Fig. 4D, solid line). Thus, high paxillin content

Fig. 4. Quantitative analysis of the association of FA molecules with FX. The quantitative analysis was performed on images of immunolabeled cells similar to those shown in Fig. 3. Motile cells were selected for this analysis, based on the time-lapse movies. Manually drawn polygons surrounding either FX at the leading edge or FA in more central positions were used to sample regions along the lamellipodium with different dynamic behavior. To evaluate the degree of localization of the various proteins in FX and in bona fide FA we used PY as an 'adhesion site marker' and calculated the degree of overlap between PY and each of the FA proteins. The results of this analysis, done on 12-19 cells, is shown for FA (A) and for FX (B). Horizontal lines indicate means. To compare the density of a particular protein (based on fluorescent labeling intensity) between FX and FA we calculated the ratio between the average intensity in FA and the average intensity in FX in the same cells (C). Bars indicate standard deviations. The intensity of PY, β_3 -integrin and talin was found to be 50% higher in FA than in FX, while paxillin, vinculin, FAK, VASP, and α -actinin are, on average, three times more intense in FA than in FX. To test the effect of local protrusion rate on the degree of co-localization of paxillin with PY in FX we measured the protrusion rate, prior to fixation, around each polygon in all the cells tested. (D) Paxillin labeling in FA (marked by \times) is not affected by the protrusion rate, whereas the labeling intensity in FX (marked by triangles) is inversely related to the protrusion rate (linear fit, solid line, $P < 0.04$).



was found in FX along slowly advancing lamella ($< 0.5 \mu\text{m}/\text{minute}$), while FX containing relatively low levels of paxillin were found in fast moving protrusions ($\sim 2.0 \mu\text{m}/\text{minute}$). The same type of analysis was carried out for talin with similar results (data not shown).

Transition of FX into zyxin-containing FA occurs during local retraction of the lamellipodium

As shown above, FX differ from FA not only in size but also in molecular composition. The most striking difference appears to be the distribution of zyxin, which is associated with FA and not with FX. Thus, the recruitment of zyxin might serve as a molecular marker for the transition of FX into FA. To monitor this process we determined the relative distributions of YFP-paxillin (used as a FX and FA marker) and CFP-zyxin (used as an exclusive FA marker) in migrating endothelial cells. The results, shown in Fig. 5 (and supplementary Movie 2: <http://jcs.biologists.org/supplemental/>), indicated that while arrays of paxillin-rich dots were formed along the base of the protruding lamellipodium, zyxin was co-localized with paxillin only in the larger, definitive FA. Interestingly, shortly (usually within 1-2 minutes) after local retraction or arrest of protrusion of the leading edge, zyxin was recruited to the FX in those areas and these adhesions underwent rapid growth. It was thus concluded that the transition of FX into FA is indeed triggered by local retraction and is manifested by local accretion of zyxin. We wondered whether this phenomenon is unique to migrating endothelial cells or is an intrinsic property of FX. To test this we have looked at cell lines of different tissue origin, (B16 melanoma, REF52 fibroblast and RCJ chondrocytes).

Fixing these cells shortly after plating and staining them for zyxin and PY revealed similar patterns of PY-positive FX at the leading edge that are devoid of zyxin (see supplementary Fig. S2: <http://jcs.biologists.org/supplemental/>).

Since one of the classical definition of FX is based on their induction by the small G-protein Rac-1 (Nobes and Hall, 1995), we further tested the molecular composition of Rac-induced FX. To this end, we transfected PAE cells with a construct encoding a constitutively active Rac-1 and immunolabeled them for actin, PY and zyxin. While retaining normal FA and SF, active Rac-1-expressing cells were more spread and accumulated numerous FX at the cell periphery. These Rac-induced FX did not contain significant levels of zyxin (Fig. 6).

The involvement of actomyosin contractility in lamellipodial protrusion and FX assembly

Is the transformation of FX into FA driven by changes in lamellipodial dynamics per se, or is it attributable to the increase in local actomyosin-driven tension? Previous studies have shown that the formation of FA and their maintenance require Rho-activated, actomyosin-based contractility (Chrzanowska-Wodnicka and Burridge, 1996; Helfman et al., 1999; Vasiliev, 1985). To determine whether these forces affect FX maturation, we have wounded endothelial cultures and treated the cells with either the Rho-associated kinase inhibitor Y27632 or with the serine/threonine kinase inhibitor H-7. Both inhibitors efficiently block Rho kinase and myosin light chain kinase activity (Narumiya et al., 2000; Uehata et al., 1997) and were previously shown to induce disassembly of FA (Riveline

Fig. 5. Recruitment of zyxin into FX occurs following local retraction of the lamellipodium. Endothelial cells were co-transfected with YFP-paxillin and CFP-zyxin. Time-lapse two-color movies captured the differential dynamic behavior of these proteins in migrating cells. A series of merged images from such a movie is presented here, where paxillin is shown in red and zyxin in green (see also Movie 2: <http://jcs.biologists.org/supplemental/>). Arrows mark the direction of leading edge movement and empty circles mark a static state. Notice that while FA contain both paxillin and zyxin (yellow) the array of FX in the advancing lamella contain paxillin alone (red). One minute after the onset of retraction (time point 5') zyxin is seen entering the FX, which grow centripetally to become FA. Scale bar: 5 μ m.

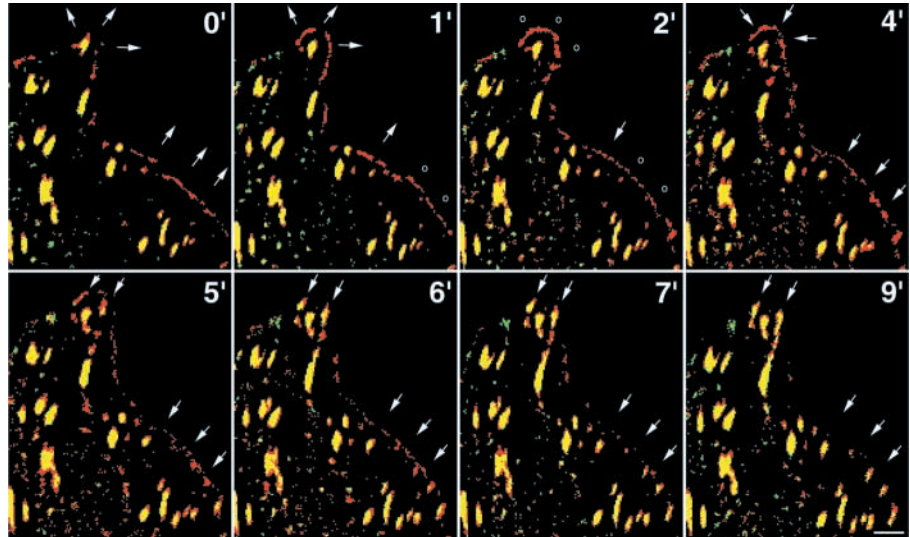
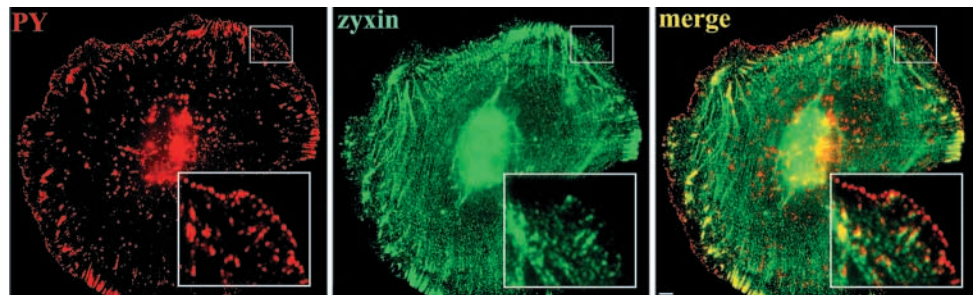


Fig. 6. FX induced by active Rac do not contain zyxin. PAE cells were transfected with dominant active Rac (L61) and double-labeled for PY and zyxin. Transfected cells have a round morphology, and while maintaining some FA they show a substantial accumulation of FX around the cell periphery, as seen by the PY staining. Zyxin is associated with FA and with actin filaments, but is absent from FX. Scale bar: 5 μ m; insets are enlargements (3 \times) of the boxed areas.



et al., 2001; Volberg et al., 1994) and accumulation of FX (Geiger and Bershadsky, 2001). Upon addition of either drug to wounded endothelial cultures, rapid spreading of the cells located at the wound edge was observed. The initial fast spreading (up to 2.5 μ m/minute) was followed by a relatively slow and uniform advancement (\sim 0.8 μ m/minute) of the leading edge and nuclei. Notably, protrusions of drug-treated cells were largely continuous, with no intermittent retractions. Prolonged treatment of the cultures resulted in complete disappearance of FA throughout the cell center and massive accumulation of FX at the cell periphery (Fig. 7). The molecular composition of these FX was similar to that described above for untreated cells. Notably, tensin and zyxin were absent from the nascent complexes, as in untreated cells. The degree of localization of the other proteins in FX was high, consistent with the rate of protrusion at the time of fixation.

Discussion

Cell locomotion is a dynamic process consisting of repeated cycles of protrusion of the leading lamella, formation of new matrix adhesions and retraction of the trailing edge (Lauffenburger and Horwitz, 1996; Small et al., 1996; Webb et al., 2002). The progression of a motile process depends on the precise regulation, in time and space, of these complex

molecular events. In this study we examined early events in the migration of endothelial cells, focusing on the formation of new matrix adhesions, namely FX, and their development into mature FA. The typical time scale for these processes ranges from a few seconds to a few minutes (see e.g. Fig. 1). To examine these processes we combined imaging of fluorescently tagged proteins in live cells with immunofluorescence microscopy of cells fixed at well-defined stages of their motile cycle.

In view of the small and transient nature of FX, it was essential to find a molecular marker for these early adhesions. As our primary molecular reference for both FA and nascent FX we have used PY, based on its high degree of overlap with β_3 -integrin in and near the lamellipodium. We realize, of course, that PY is not unique to adhesion sites and is also diffusely distributed along the plasma membrane or in the cytoplasm as well as in membrane vesicles. However, our preliminary experiments indicated that all the β_3 -integrin-containing dots were tyrosine phosphorylated, and $>80\%$ of the PY-labeled dots in the leading edge also contain β_3 -integrin.

We show that integrin- and PY-rich FX are formed immediately behind a 1-2 μ m wide area at the front of the lamellipodium, where active actin polymerization takes place. The cytoskeletal proteins that co-localize with actin in this region, include VASP (Bear et al., 2000; Rottner et al., 1999a),

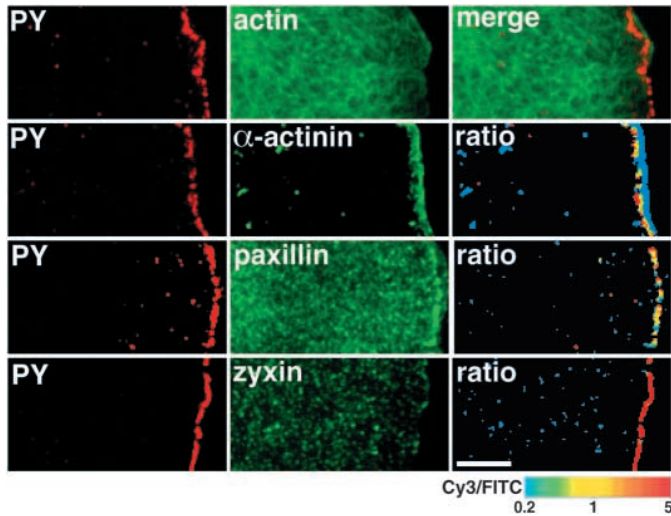


Fig. 7. Inhibition of contractility using the Rho-kinase inhibitor Y27632, leads to the disappearance of FA and an enrichment of zyxin-free FX. Migrating endothelial cells were treated for 30 minutes with Y27632 or with the serine/threonine kinase inhibitor H-7 (not shown), which block acto-myosin contractility. FA and SF disappear following this treatment, and at the cell periphery, behind an exceptionally broad α -actinin-rich band, a conspicuous array of FX is apparent. These FX contain paxillin, but not zyxin. Scale bar: 5 μ m.

α -actinin (Laukaitis et al., 2001), scar/WAVE (Hahne et al., 2001), and Abl interactor proteins (Stradal et al., 2001).

Quantitative analysis of the time-dependent recruitment of several FA proteins to newly formed FX, revealed an ordered entry of these molecules to newly formed adhesions (Fig. 4B). The earliest FX detected under a protruding lamella contain $\alpha_v\beta_3$ -integrin, and are tyrosine phosphorylated. Talin and paxillin are, apparently, incorporated next, followed by vinculin and FAK. VASP and α -actinin were present only in some of the FX (~25%), while tensin and zyxin were essentially absent from these sites.

Hierarchical assembly of FA has been described previously using immunofluorescent labeling of locomoting fish keratocytes (Lee and Jacobson, 1997) and using migrating CHO cells expressing different GFP fusion proteins (Laukaitis et al., 2001). In the former study, close contacts in the front of the keratocyte lamellipodium were shown to contain β_1 -integrin and talin. Somewhat older contacts contained vinculin, and those located at more posterior regions contain α -actinin and FAK. Interestingly, in the fish keratocyte, paxillin was found only in large FA and was largely absent from the leading edge (Lee and Jacobson, 1997). Laukaitis et al., in contrast, found paxillin to be the first protein recruited to FX, followed by α -actinin, and later by α_5 -integrin (Laukaitis et al., 2001). The difference between our observation showing early entry of integrin into complexes and the claim that integrin is a 'late comer' can be explained by the difference in the integrin type examined (e.g. $\alpha_v\beta_3$ versus $\alpha_5\beta_1$). Our observation is in line with reports on the presence of high-affinity $\alpha_v\beta_3$ -integrin at the leading edge of endothelial cells following activation with Rac-1 (Kiosses et al., 2001). A recent report, in which the force generated by a minimal matrix-integrin-cytoskeleton connection was measured in normal and talin knockout cells,

has suggested that talin is an early essential component of the adhesion complex, in agreement with our data (Jiang et al., 2003).

It is interesting to note that molecules that have been implicated in regulating cell migration, such as zyxin (Drees et al., 1999), tensin (Chen et al., 2002) and FAK (Ilic et al., 1995; Owen et al., 1999), are hardly detectable in FX of migrating endothelial cells. It is not clear whether this is the result of cell-type-specific variability or of differences in the definition of FX, namely, the reference to small but definitive (i.e. – zyxin-containing) FA in retracting areas as FX. Rottner et al. (Rottner et al., 2001), who first introduced GFP-tagged zyxin into cells, did not detect zyxin co-localized with VASP at lamellipodial tips in migrating B16 melanoma cells or in REF cells injected with a fluorescent zyxin antibody. Nix et al. (Nix et al., 2001), however, reported its localization in the leading edge of Vero cells. We have examined several cell types and failed to detect any zyxin in FX formed under a protruding lamella (supplemental Fig. S2: <http://jcs.biologists.org/supplemental/>). Another intriguing observation is the late entry and low levels of FAK in FX, in contrast to the early and intense phosphorylation of these structures. This finding suggests that either another tyrosine-specific kinase, such as Fyn (von Wichert et al., 2003), is involved in the early phosphorylation events, or that proteins that are phosphorylated elsewhere are recruited to these sites. The present study highlights not only the process of FX formation, but also their transformation into FA. In fact – we propose here a new differential definition for the two structures. We show that FX gradually accumulate different adhesion-associated molecules, yet their final composition is still different from that of bona-fide FA (compare Fig. 4A with 4B). In addition, the local concentration of the different proteins and PY in FX, as measured by average intensity following immunolabeling, does not exceed 35%–65% of the values measured for the corresponding molecule at nearby FA (Fig. 4C). Thus, it appears that FX do not just gradually 'mature' into FA, but that a specific stimulus is needed for their transition into FA.

We present here evidence that such stimulus is triggered by local retraction of the lamellipodium. Thus, while FX are formed under protruding lamella, FA, with their full repertoire of components, develop only at retracting regions. The most conspicuous event that characterized this transition is the recruitment of zyxin to the adhesion site (Fig. 5). This observation provides the first molecular hallmark that unequivocally distinguishes between the two structures. The association between lamellipodial retraction and transformation of low density GFP- β_3 -integrin adhesions into high density adhesion sites has been noted by Ballestrem et al. (Ballestrem et al., 2001) in 3T3 cells, and the link between retraction and FA growth has been described by Vasiliev (Vasiliev, 1985) nearly twenty years ago. Apparently, acto-myosin-driven forces applied to the adhesion site further activate local tyrosine phosphorylation, induce local increase in the levels of FA molecules, and recruit new proteins, such as zyxin, α -actinin and tensin, which are abundant in FA but not in FX. The fact that contraction is necessary for FX to develop into FA implies that loss of tension might block this transition and lead to accumulation of FX. Indeed, cells treated with the Rho kinase inhibitor Y27632, accumulate FX under

the lamellipodium (Fig. 7), in agreement with the findings of Ballestrem et al., (Ballestrem et al., 2001) (see also Geiger and Bershadsky, 2001).

The close proximity between FX, which are rather stationary structures (Fig. 1) (see Ballestrem et al., 2001), and the highly dynamic leading edge has some intriguing implications for the mechanism of cell motility that should be considered here. Recent studies have shown that the forward protrusion of the lamellipodium is driven by actin polymerization, in which new monomers are added at the barbed end of the actin filament, either at the very edge of the lamella or at Arp2/3-mediated branches (reviewed by Borisy and Svitkina, 2000). In order to push the membrane forward and produce a protrusion, these growing filaments must be immobilized at more posterior positions. The formation of an array of actin- and matrix-bound FX at the base of the lamellipodium could provide such a physical anchor. This mechanism could, in principle, act as a molecular 'gear box', whereby controlled transmembrane tethering of the centripetally expanding actin mesh to the extracellular matrix or to stationary cytoskeletal elements, supports a local protrusion of the lamellipodium.

The other side of the same coin is the involvement of actin polymerization in the nearby lamellipodium in the regulation of FX formation and turnover. As pointed out here and elsewhere (Ballestrem et al., 2001; Kaverina et al., 2002; Laukaitis et al., 2001; Rottner et al., 1999a; Rottner et al., 1999b) the majority of FX have a rather short life span, and they disassemble within minutes after their formation. FX form along the leading edge, and when the edge advances, those FX that are 'left behind' disassemble while new ones are formed in more anterior locations. The intriguing mechanism underlying FX instability, and the involvement of actin polymerization in this process are still poorly understood.

In conclusion, the present study reveals a complex and highly coordinated relationship between the different processes involved in cell locomotion, in which FX play a key role. They are formed during the forward movement of the lamella – sequentially recruiting different FA molecules – and transform into FA during the retraction phase. Zyxin is shown here to be a hallmark of this retraction-dependent transformation of FX into stable FA.

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References

- Ballestrem, C., Hinz, B., Imhof, B. A. and Wehrle-Haller, B. (2001). Marching at the front and dragging behind: differential alpha5beta3-integrin turnover regulates focal adhesion behavior. *J. Cell Biol.* **155**, 1319-1332.
- Bear, J. E., Loureiro, J. J., Libova, I., Fassler, R., Wehland, J. and Gertler, F. B. (2000). Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell* **101**, 717-728.
- Borisy, G. G. and Svitkina, T. M. (2000). Actin machinery: pushing the envelope. *Curr. Opin. Cell Biol.* **12**, 104-112.
- Chen, H., Duncan, I. C., Bozorgchami, H. and Lo, S. H. (2002). Tensin1 and a previously undocumented family member, tensin2, positively regulate cell migration. *Proc. Natl. Acad. Sci. USA* **99**, 733-738.
- Chrzanowska-Wodnicka, M. and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403-1415.
- Critchley, D. R. (2000). Focal adhesions – the cytoskeletal connection. *Curr. Opin. Cell Biol.* **12**, 133-139.
- DeMali, K. A., Barlow, C. A. and Burridge, K. (2002). Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. *J. Cell Biol.* **159**, 881-891.
- Drees, B. E., Andrews, K. M. and Beckerle, M. C. (1999). Molecular dissection of zyxin function reveals its involvement in cell motility. *J. Cell Biol.* **147**, 1549-1560.
- Galbraith, C. G., Yamada, K. M. and Sheetz, M. P. (2002). The relationship between force and focal complex development. *J. Cell Biol.* **159**, 695-705.
- Geiger, B. and Bershadsky, A. (2001). Assembly and mechanosensory function of focal contacts. *Curr. Opin. Cell Biol.* **13**, 584-592.
- Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K. M. (2001). Transmembrane extracellular matrix–cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* **2**, 793-805.
- Hahne, P., Sechi, A., Benesch, S. and Small, J. V. (2001). Scar/WAVE is localised at the tips of protruding lamellipodia in living cells. *FEBS Lett.* **492**, 215-220.
- Helfman, D. M., Levy, E. T., Berthier, C., Shtutman, M., Riveline, D., Grosheva, I., Lachish-Zalait, A., Elbaum, M. and Bershadsky, A. D. (1999). Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. *Mol. Biol. Cell* **10**, 3097-3112.
- Huttenlocher, A., Ginsberg, M. and Horwitz, A. (1996). Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. *J. Cell Biol.* **134**, 1551-1562.
- Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.
- Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539-544.
- Jiang, G., Giannone, G., Critchley, D. R., Fukumoto, E. and Sheetz, M. P. (2003). Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature* **424**, 334-337.
- Kaverina, I., Krylyshkina, O. and Small, J. V. (2002). Regulation of substrate adhesion dynamics during cell motility. *Int. J. Biochem. Cell Biol.* **34**, 746-761.
- Kiosses, W. B., Shattil, S. J., Pampori, N. and Schwartz, M. A. (2001). Rac recruits high-affinity integrin alpha5beta3 to lamellipodia in endothelial cell migration. *Nat. Cell Biol.* **3**, 316-320.
- Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.
- Laukaitis, C. M., Webb, D. J., Donais, K. and Horwitz, A. F. (2001). Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. *J. Cell Biol.* **153**, 1427-1440.
- Lee, J. and Jacobson, K. (1997). The composition and dynamics of cell-substratum adhesions in locomoting fish keratocytes. *J. Cell Sci.* **110**, 2833-2844.
- Martin, K. H., Slack, J. K., Boerner, S. A., Martin, C. C. and Parsons, J. T. (2002). Integrin connections map: to infinity and beyond. *Science* **296**, 1652-1653.
- Narumiya, S., Ishizaki, T. and Uehata, M. (2000). Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol.* **325**, 273-284.
- Nix, D. A., Fradelizi, J., Bockholt, S., Menichi, B., Louvard, D., Friederich, E. and Beckerle, M. (2001). Targeting of zyxin to sites of actin membrane interaction and to the nucleus. *J. Biol. Chem.* **276**, 34759-34767.
- Nobes, C. D. and Hall, A. (1995). Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem. Soc. Trans.* **23**, 456-459.
- Owen, J. D., Ruest, P. J., Fry, D. W. and Hanks, S. K. (1999). Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Mol. Cell Biol.* **19**, 4806-4818.
- Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C. and Yamada, K. M. (2000). Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. *J. Cell Biol.* **148**, 1075-1090.
- Petit, V. and Thiery, J. P. (2000). Focal adhesions: structure and dynamics. *Biol. Cell* **92**, 477-494.

- Riveline, D., Zamir, E., Balaban, N. Q., Schwarz, U. S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. and Bershadsky, A. D.** (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDial-dependent and ROCK-independent mechanism. *J. Cell Biol.* **153**, 1175-1186.
- Rottner, K., Behrendt, B., Small, J. V. and Wehland, J.** (1999a). VASP dynamics during lamellipodia protrusion. *Nat. Cell Biol.* **1**, 321-322.
- Rottner, K., Hall, A. and Small, J. V.** (1999b). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* **9**, 640-648.
- Rottner, K., Krause, M., Gimona, M., Small, J. V. and Wehland, J.** (2001). Zyxin is not colocalized with vasodilator-stimulated phosphoprotein (VASP) at lamellipodial tips and exhibits different dynamics to vinculin, paxillin and VASP in focal adhesions. *Mol. Biol. Cell* **12**, 3103-3113.
- Small, J. V., Anderson, K. and Rottner, K.** (1996). Actin and the coordination of protrusion, attachment and retraction in cell crawling. *Biosci. Rep.* **16**, 351-368.
- Stradal, T., Courtney, K. D., Rottner, K., Hahne, P., Small, J. V. and Pendergast, A. M.** (2001). The Abl interactor proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia. *Curr. Biol.* **11**, 891-895.
- Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. et al.** (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990-994.
- Vasiliev, J. M.** (1985). Spreading of non-transformed and transformed cells. *Biochim. Biophys. Acta* **780**, 21-65.
- Volberg, T., Geiger, B., Citi, S. and Bershadsky, A.** (1994). Effect of protein kinase inhibitor H-7 on the contractility, integrity, and membrane anchorage of the microfilament system. *Cell Motil. Cytoskel.* **29**, 321-338.
- von Wichert, G., Jiang, G., Kostic, A., De Vos, K., Sap, J. and Sheetz, M. P.** (2003). RPTP-alpha acts as a transducer of mechanical force on alphav/beta3-integrin-cytoskeleton linkages. *J. Cell Biol.* **161**, 143-153.
- Webb, D. J., Parsons, J. T. and Horwitz, A. F.** (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nat. Cell Biol.* **4**, E97-E100.
- Zamir, E. and Geiger, B.** (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* **114**, 3583-3590.
- Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B. and Kam, Z.** (1999). Molecular diversity of cell-matrix adhesions. *J. Cell Sci.* **112**, 1655-1669.
- Zamir, E., Katz, M., Posen, Y., Erez, N., Yamada, K. M., Katz, B. Z., Lin, S., Lin, D. C., Bershadsky, A., Kam, Z. et al.** (2000). Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat. Cell Biol.* **2**, 191-196.