Focal Adhesion Motility Revealed in Stationary Fibroblasts

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Focal adhesions (FAs) are clustered integrins and associated proteins that mediate cell adhesion and signaling. A green fluorescent protein–β1 integrin chimera was used to label FAs in living cells. In stationary cells, FAs were highly motile, moving linearly for several plaque lengths toward the cell center. FA motility was independent of cell density and resulted from contraction of associated actin fibers. In migrating cells, FAs were stationary and only moved in the tail. FA motility in stationary cells suggests that cell movement may be regulated by a clutch-like mechanism by which the affinity of integrins to substrate may be altered in response to migratory cues.

Adhesive contacts between cells and the substratum are critical for cell spreading and migration mediated by integrins (*1*). In fibroblasts, these integrins concentrate in specific regions within the plasma membrane, called FAs, where actin stress fibers and associated proteins are anchored (*2*). During fibroblast migration, FAs form at the leading edge of the cell, remain fixed as the cell migrates over them, and then detach when they are at the rear of the cell (*3*). The mechanisms that regulate polarized FA formation and detachment in migrating cells are largely unknown.

To study FAs in living cells, we generated a chimera of green fluorescent protein (GFP), the transmembrane and cytoplasmic domains of the β1 integrin subunit, and the signal sequence from the α1 integrin subunit, such that the GFP region would be extracellular (Fig. 1A). Stable cell lines expressing low levels of GFP-integrin were selected to limit the effect of the chimera on integrin function (*4*–*6*). The GFP-integrin labeled all FAs, as shown by colocalization with endogenous integrins (Fig. 1D) and the FA marker vinculin (*7*). The GFP-integrin cell lines were similar to the parental cell line in morphology (Fig. 1B), adhesion to fibronectin (Fig. 1C), growth (*7*), and spreading on fibronectin (*7*); hence, the chimera had no detectable effect on the cell's adhesive properties.

In stationary cells, FAs labeled with GFP-integrin appeared motile (Fig. 2). When different colors are used to represent sequential frames from a time-lapse recording (Fig. 2, A and B), moving FAs appear as rainbows in overlay images. These movements were linear, usually occurred without change in FA area or shape, occurred relative to the substratum and cell edge, and involved distances of greater than one FA length. The motile FAs were distributed throughout the cell; most moved toward the cell center, but some moved along the cell edge. New FAs formed near the cell edge as others moved inward. FAs infrequently split in two or elongated during movement. The rate of movement was 0.12 ± 0.08 μm/min (n = 128 FAs; 9 cells), similar to the rate of 3T3 cell migration (*8*). We defined a motile FA as one that moved at least one focal adhesion plaque length within 1 hour. By this criterion, 65 ± 27% (n = 692 FAs; 10 cells) of the FAs in individual cells were motile. Variability in FA motility may reflect differences in the cell cycle, metabolic activity, or local substratum conditions. Similar FA movements occurred in stationary cells within a monolayer, at the edge of a wounded monolayer, and in well-spread cells in sparse cultures; these observations suggest that FA movement is independent of cell density and cell-cell interactions.

In contrast, little movement of GFP-integrin–labeled FAs was observed in migrating cells stimulated to migrate by wounding a monolayer or after cell division. Color overlays of three sequential images indicated that the vast majority of FAs in a migrating cell do not move (Fig. 2D): FAs were solid colors rather than a rainbow detected in the stationary cells (see Fig. 2C). Four zones of FA behavior were discerned in migrating cells: (i) a formation zone at the front ~1/4 of the cell (FAs purple); (ii) a persistence zone between the formation zone and the nucleus (most FAs white due to overlapping purple, red, and green); (iii) a culling zone between the nucleus and the tail where some FAs were lost (FAs yellow due to overlapping red and green) while others were maintained (FAs white); and (iv) a small motile zone in the tail (FAs rainbows of red and green). Hence, FAs first form at the front of a migrating cell and do not move as the cell migrates over them, although some of them are lost (culling zone). Only in the tail do FAs begin to move. These results corroborate previous studies on FAs in migrating cells (*3*) but define zones of differential FA regulation.

The role of actin in FA movement was explored by microinjection of rhodamine-ac-

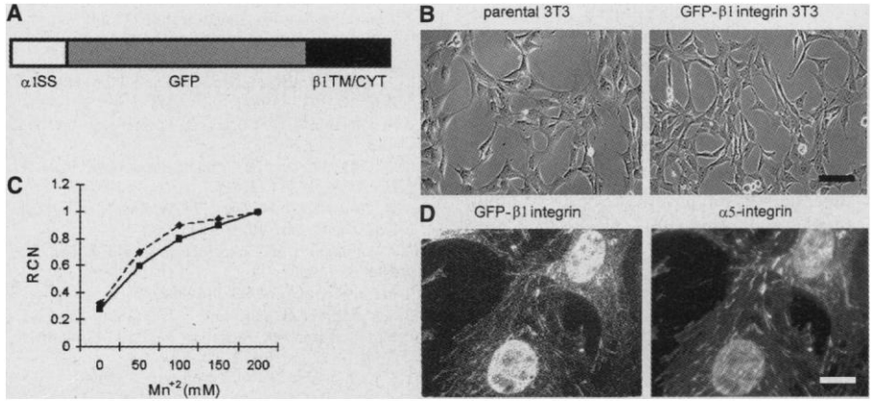

Fig. 1. Characterization of the GFP-integrin chimera. (**A**) Diagram showing the signal sequence from α1 integrin (α1SS) fused to GFP and transmembrane and cytoplasmic domains of β1 integrin (β1TM/CYT) (*14*). (**B**) Phase-contrast images of parental 3T3 cells and GFP-integrin–expressing cells. Scale bar, 60 μm. (**C**) Mn^{2+}-activated adhesion of parental cells (solid line) and GFP-integrin–expressing cells (dashed line). Cells were plated on fibronectin-coated dishes in increasing concentrations of Mn^{2+} and assayed for relative cell number (RCN) (*5*). (**D**) GFP-integrin located in FAs. GFP-integrin–expressing cells were fixed and immunostained with antibodies to GFP (left) and to α5 integrin (right) (*17*). The nuclear staining with antibody to GFP is likely an artifact, because it was not observed by direct GFP fluorescence (Figs. 2 and 3). Scale bar, 10 μm.

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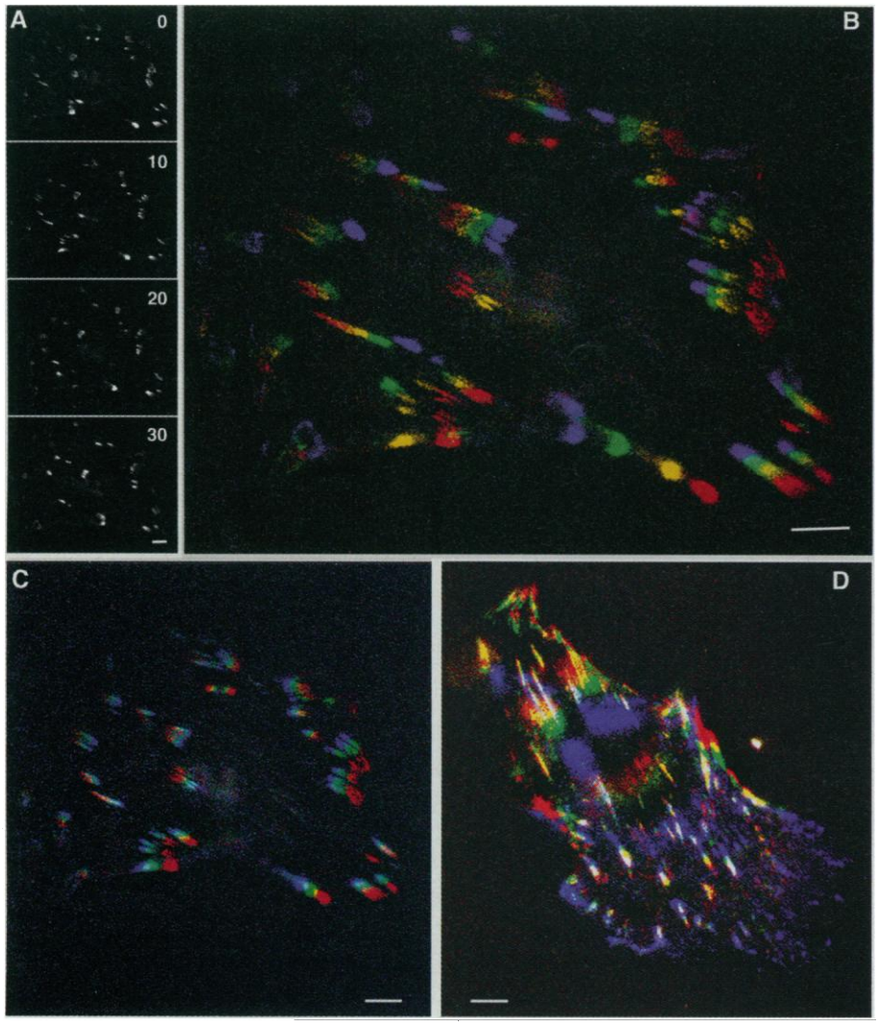

Fig. 2. GFP-integrin dynamics in stationary and migrating cells. (**A**) Fluorescence images from a time-lapse sequence (0, 10, 20, and 35 min) of GFP-integrin dynamics in a stationary cell. (**B**) Four-color overlay of images depicted in (A) to show movement of the FAs in stationary cells. Images taken at 0, 10, 20, and 35 min were encoded red, yellow, green, and purple, respectively. [See also movie 1 (*18*).] (**C**) Three-color overlay of images taken at 0, 10, and 20 min (encoded red, green, and purple, respectively) from a recording of a stationary cell. (**D**) Three-color overlay of images taken at 0, 10, and 20 min [same color codes as in (C)] from a recording of a migrating cell after cell division. FAs may appear as a single color (purple, white, or yellow) rather than a rainbow of different colors, except for FAs in the tail, which are rainbows of red and green. Scale bars, 5 μm.

tin (R-actin) into stationary cells, followed by simultaneous imaging of rhodamine and GFP fluorescence. All GFP-labeled FAs had associated actin fibers (Fig. 3, A and C). While FAs moved, they remained associated with actin fibers. Actin fibers with FAs on both ends (bipolar fibers) (Fig. 3B) shortened as the FAs moved toward each other (fibers did not appear on the trailing edge of the FAs). Hence, actin contraction may be important in FA movement. By staining fixed cells with R-phalloidin after recording FA dynamics, we observed moving FAs connected to monopolar fibers (Fig. 3C). Thus, formation of bipolar actin fibers is not a prerequisite for FA movement.

Isotonic contraction of the associated actin fiber may be responsible for the movement of FAs in stationary cells. Consistent with this, an inhibitor of myosin contraction, 2,3-butanedione monoxoime (*9*), blocked movement of FAs (*7*). Conversely, 10 μM nocodazole, which depolymerizes microtubules and stimulates contractility by activating myosin (*10*), increased the rate of FA movement in stationary cells fourfold to 0.57 ± 0.28 μm/min (n = 31 FAs; 3 cells). Taxol, which stabilizes microtubules, had no effect. These results suggest that contraction of actin fibers is responsible for FA motility.

Interference reflection microscopy (IRM) can estimate the distance between the ventral cell surface and the substratum. Portions of the cell within 15 nm of the substratum, as is the case with FAs, appear as dark contrast against a gray background in the zero-order IRM image (*1*). The patterns of GFP fluorescence and IRM contrast in stationary cells were closely matched, indicating that GFP-labeled FAs were closely apposed to the substratum (Fig. 4, A and B). When FAs moved, they maintained close apposition to the substratum (dark contrast by IRM) (Fig. 4, A and B; FAs 1, 3, and 4). However, for some fast-moving FAs, we observed a transient diminution of

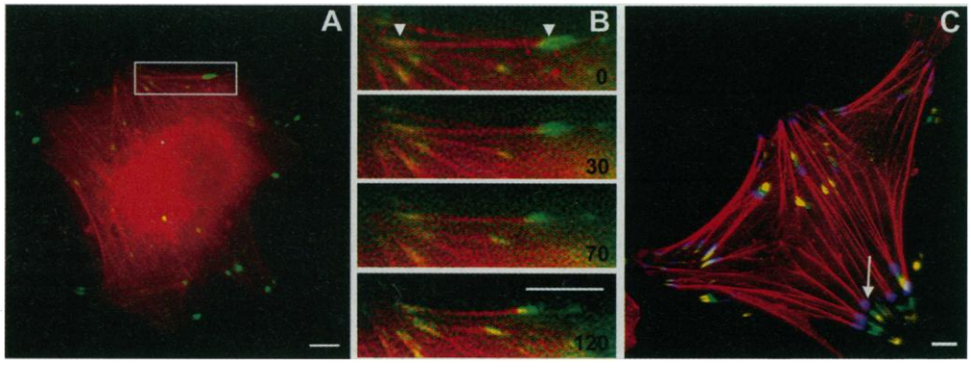

Fig. 3. Moving FAs remain associated with actin filaments. (**A** and **B**) Cells expressing GFP-integrin were microinjected with R-actin, and GFP and rhodamine fluorescence were imaged (*19*). In (A), an image of a cell microinjected with R-actin (red) is overlaid on the GFP-integrin fluorescence (green). In (B), images from the time-lapse recording (taken at 0, 30, 70, and 120 min) of the boxed area in (A) are shown. Two FAs (arrowheads) at the end of an actin stress fiber move toward each other without losing connection to the stress fiber. [See also movie 2 (*18*).] (**C**) Composite image of GFP-integrin–labeled FAs (green) in a living cell overlaid on the F-actin image revealed by R-phalloidin staining (red) of the same cell after fixation. The GFP image was formed by color-coding images (8-min intervals) from the time-lapse recording (yellow for the earliest time, followed by green, then purple). Some FAs appear outside the cell because of cell shape changes. Arrow indicates a moving FA connected to a monopolar actin fiber. Scale bars, 5 μm.

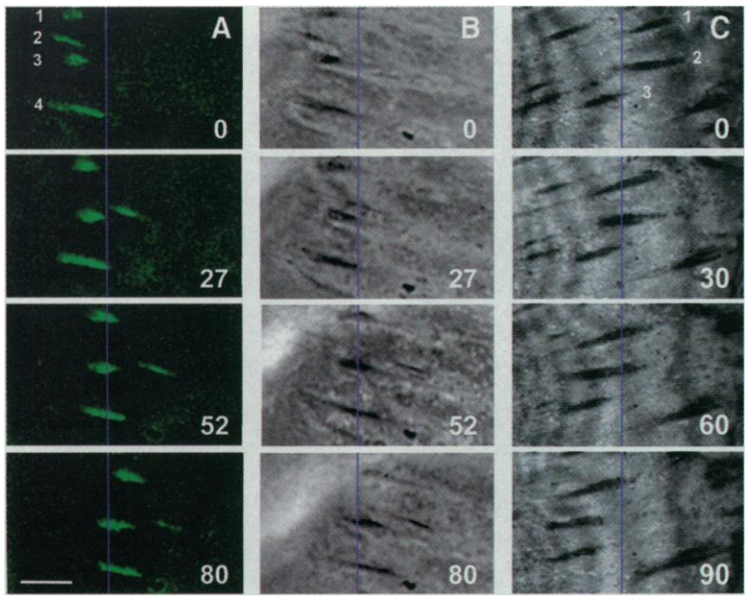

Fig. 4. Moving FAs in stationary GFP-integrin cells and stationary parental 3T3 cells remain in close contact with the substratum. **(A)** Fluorescence images from a time-lapse recording of a cell expressing GFP-integrin (taken at 0, 27, 52, and 80 min), showing four moving FAs detectable by GFP fluorescence (blue lines are a fiduciary mark) (*20*). **(B)** IRM images of a cell expressing GFP-integrin, corresponding to the fluorescence images shown in (A); FAs are black (*20*). [See also movie 3 (*18*).] **(C)** IRM images from a time-lapse recording (taken at 0, 30, 60, and 90 min) showing three moving FAs in a parental 3T3 cell. Scale bars, 2.5 μm.

IRM contrast (Fig. 4, A and B; FA 2). Hence, although most moving FAs remained closely apposed to the substratum, interaction with the substratum was reduced for some rapidly moving FAs.

Using IRM, similar FA movements were observed in stationary parental 3T3 cells (Fig. 4C), as well as in stationary rat NRK fibroblasts and diploid human fibroblasts (*7*). In parental 3T3 cells, the rate and extent of FA movement was similar to that in cells expressing GFP-integrin: 0.10 ± 0.04 μm/min (n = 78 FAs; 4 cells) and 67 ± 17%, respectively (n = 370 FAs; 7 cells). Hence, FA movement is characteristic of stationary cells and is not induced by the GFP-integrin.

The extensive FA movement in stationary cells we have described is clearly different from the restricted movements of FAs observed in the tail of migrating cells [this study and (*3*)], localized remodeling of FAs (*3*), or movements of integrins attached to beads (*11*). Because FAs provide sites of adhesion for migration, the movement of FAs in stationary cells may prevent the generation of the traction necessary for migration. Upon stimulation of cell migration, FAs must be regulated so that most remain fixed to the substratum. By maintaining FAs in a motile "idling" state, cells may be able to rapidly polarize toward migratory cues by selecting stronger adhesion sites to provide traction and allowing unselected, motile FAs to turn over.

That FAs exhibit nonmotile and motile states coordinated with cell migration suggests the existence of a "molecular clutch" to alternate between these states. The transition between these states must reflect the balance between tension and adhesion. The molecular clutch may regulate either the affinity of the integrin for the extracellular matrix (ECM) or the tension applied to the FA by the actin cytoskeleton, or both. Regulation of integrin affinity has been noted previously, and there is an optimum affinity at which cells are capable of migrating (*12*). However, neither high substrate concentrations of fibronectin nor addition of Mn^{2+} blocked FA motility (*7*). These conditions increase binding in cell adhesion assays, but it is unclear whether they increase integrin affinity under our conditions. Regulation of tension by altering actin contraction is known to be mediated by factors such as Rho and Ca^{2+} (*13*), and our results with nocodazole suggest that changes in tension can alter the velocity of FA movements. Increasing tension without altering FA affinity for the ECM may decrease cell traction and may also contribute to FA movements that are involved in remodeling the ECM, although ECM remodeling is currently thought to occur on a much longer time scale. Whatever the composition of the molecular clutch, the existence of moving FAs in stationary cells shows directly that a cell is able to regulate its interactions with the ECM in a previously unexpected fashion.

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6. NIH 3T3 cells were stably transfected with pLen GFP-β1 (*14*) and pSVneo as described (*5*). Cell lines expressing low levels of GFP–β1 integrin were characterized by flow cytometry using methods described in (*5*). We confirmed that GFP was extracellular by labeling with antibodies to GFP.
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14. The GFP–β1 integrin chimera was constructed in pLen (*5*) using standard recombinant DNA methods. The chimera consisted of the 28–amino acid signal sequence of α1 integrin, S65T GFP, and cytoplasmic and transmembrane domains of chicken β1 integrin. The construct was generated by overlap PCR (*5*) using the following sequences: α1 signal sequence/GFP, 5′-TTCTCCTTTACTGGAGACGCAGAAGCCTAG-3′; GFP/β1 transmembrane, 5′-AATGGGTATGATTTTGTATAGTTCATCCAT-3′.
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16. Cells were assembled into chambers and imaged as described (*15*) using a Nikon Eclipse TE300 microscope maintained at 37°C. A Nikon HQ fluorescein filter cube was used for GFP fluorescence. Fluorescence and phase-contrast images were recorded with a cooled charge-coupled device (CCD) camera with a back-illuminated chip (Princeton Instruments, Trenton, NJ) and Metamorph software. Sequential images were taken every 1 to 4 min.
17. Cells were plated on fibronectin-coated cover slips, fixed, and stained as described (*5*). Rabbit antibody to GFP was from Clontech, mouse antibody to integrin α5 was from Pharmingen, and anti-vinculin was purchased from Sigma. The secondary antibodies were from Cappel (Durham, NC).
18. For movies, see *Science* Online (www.sciencemag.org/feature/data.1042403.shl).
19. R-actin (2 mg/ml; Cytoskeleton, Denver, CO) was microinjected into cells expressing GFP-integrin and allowed to incorporate for 2 to 4 hours before recording simultaneous GFP and rhodamine fluorescence (*16*). Sequential images were taken every 4 to 8 min.
20. IRM was done using a Nikon 100× Fluor objective with an iris. A green filter was used to eliminate higher-ordered reflections and to prevent excitation of GFP. IRM and GFP fluorescence images were recorded consecutively with the cooled CCD once every minute as described (*16*).
21. We thank T. Salmon and S. Inoue for stimulating discussions, J. Larson and P. Bent (Nikon) for help with microscopes, and R. Y. Tsien for S65T GFP. A.M. and R.J.P. were supported by National Institute on Aging and National Institute of General Medical Sciences training grants, respectively. Some experiments were done while G.G.G. was a Nikon Fellow at the Marine Biological Laboratories in Woods Hole, MA. Supported by NIH grants GM44585 (E.E.M.) and GM42026 (G.G.G.).

7 June 1999; accepted 30 August 1999

References and Notes

[8] **Enhanced Motility in NIH 3T3 Fibroblasts That Overexpress Gelsolin**

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Science, New Series, Vol. 251, No. 4998. (Mar. 8, 1991), pp. 1233-1236.

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