The spreading of motile cells on a substrate surface is accompanied by reorganization of their actin network. We show that spreading in the highly motile cells of Dictyostelium is non-monotonic, and thus differs from the passage of spreading cells through a regular series of stages. Quantification of the gain and loss of contact area revealed fluctuating forces of protrusion and retraction that dominate the interaction of Dictyostelium cells with a substrate. The molecular basis of these fluctuations is elucidated by dual-fluorescence labeling of filamentous actin together with proteins that highlight specific activities in the actin system. Front-to-tail polarity is established by the sorting out of myosin-II from regions where dense actin assemblies are accumulating. Myosin-IB identifies protruding front regions, and the Arp2/3 complex localizes to lamellipodia protruded from the fronts. Coronin is used as a sensitive indicator of actin disassembly to visualize the delicate balance of polymerization and depolymerization in spreading cells. Short-lived actin patches that co-localize with clathrin suggest that membrane internalization occurs even when the substrate-attached cell surface expands. We conclude that non-monotonic cell spreading is characterized by spatiotemporal patterns formed by motor proteins together with regulatory proteins that either promote or terminate actin polymerization on the scale of seconds.

Introduction

Attachment of a motile cell to an adhesive surface results in restructuring of the actin network in the cell cortex. As a consequence the cell will spread, and subsequently migrate provided the forces generated in the cytoskeleton counterbalance cell-to-substrate adhesion. The dynamics and mechanisms of cell spreading are dependent on cell type and culture conditions.

The dynamics of spreading has been analyzed most comprehensively in mouse embryonic fibroblasts using total internal reflection fluorescence (TIRF) microscopy as a technique to quantify the growth of a contact area. Depending on the culture conditions, the fibroblasts exhibit one of two modes of spreading on fibronectin-coated surfaces: anisotropic spreading in the presence of serum, and isotropic spreading under serum-deprived conditions. The anisotropic spreading is characterized by stochastic steps of lamellipod extension that are interrupted by periods of quiescence or retraction. Isotropic spreading can be separated into three phases distinguished by the power-law dependence of growth of the contact area. (1) In the basal phase, the area growth is small. (2) The following phase of fast, uninterrupted spreading is characterized by a high rate of actin polymerization at the leading edge of the lamellipodial sheet surrounding the cell. (3) In the final phase, continuous expansion of the contact area turns into oscillatory growth, which means that periods of expansion alternate with periods of contraction. The contractions are mediated by myosin-II, the activity of which is regulated by myosin light-chain kinase.

In fibroblasts and other cells which spread by lamellipod expansion, actin filaments play an active role in pushing the leading edge forward. Quite in contrast, in the spreading of HeLa cells the cortical actin network is thought to play only a passive role as a highly viscous shell. In this case, the actin layer limits the speed of spreading, as inferred from the accelerated spreading in the presence of cytochalasin D.

Chamaraux et al. concentrated on an initial phase of spreading in Dictyostelium cells. About half of the cells in a population were observed to spread in a monotonic manner before the contact area reached an average size. Subsequently, the area fluctuated around this size when the cells became motile. Although the early spreading dynamics resembles that of HeLa cells, as pointed out by Cuvelier et al., Dictyostelium cells do not spread by deformation of an adhesive viscous shell. Actin polymerization is considered to be the driving force, since depolymerization by cytochalasin decreases the spreading kinetics to one tenth of its normal value. The spreading has been quantitatively modeled assuming that it depends on the rate of actin polymerization and comes to a halt when membrane tension counteracts polymerization at the cell border.

In the present study we have exposed the highly motile cells of Dictyostelium discoideum to surfaces of varying adhesiveness, and used TIRF and high-speed confocal scanning microscopy in order to selectively record structures built by actin and associated proteins in the substrate-apposed cortex of the cells. In studying spreading in these cells, we find continuous fluctuations in the organization and activity of the actin system, which are associated with the gain and loss

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**Research Paper**

**Actin-cytoskeleton dynamics in non-monotonic cell spreading**

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of contact area. These fluctuations commence immediately after the first contact of a cell with the substrate. We argue that Dictyostelium undergoes non-monotonic spreading, a mode of cell-substrate interaction characterized by the cell-autonomous generation of patterns in the structure and activity of the actin system.

By tagging proteins with mRFP or GFP we combined a label for filamentous actin, a deletion construct (LimEΔ) of the Dictyostelium LimE protein, with (1) the double-headed myosin-II, which serves as a marker for a retracting tail, (2) myosin-IB (myoB), one of the single-headed motor proteins that associate with the leading edge of a cell, (3) the Arp2/3 complex, the nucleator of dendritic actin structures, primarily localizing to lamellipodia, and (4) coronin, a WD40-repeat protein that is recruited to sites of actin depolymerization. In addition, we show that the majority of actin patches at the substrate-attached surface are associated with clathrin, indicating that these patches are involved in membrane recycling rather than in cell-to-substrate adhesion.

All the proteins employed in this study are common constituents or regulators of the actin system in motile eukaryotic cells. Myosin-II is the only conventional myosin in D. discoideum, and is the myosin responsible for posterior contraction and generation of a pushing force for the anterior extension of a cell. The activity of myosin-II in Dictyostelium is tuned by heavy-chain phosphorylation: dephosphorylation of three threonine residues on the tail of myosin-II enables the protein to associate into bipolar filaments, which can contract a network of actin filaments. MyoB is distinguished, among the twelve unconventional myosins in D. discoideum, by its abundance and prominent function in cell motility, phagocytosis, and response to chemotactant. Nevertheless, it shares specific functions with one or several of the myosin-I isoforms myoA, myoD and myoF. MyoB associates specifically with the plasma membrane and is connected through a linker protein, CARMIL, to the Arp2/3 complex. This heptameric complex mediates nucleation and branching of actin filaments in motile cells, and is activated in response to external signals through proteins of the WASP/WAVE family. Coronin has initially been described as a constituent of crown-shaped protrusions of the cell surface and as a protein involved in cytokinesis, phagocytosis and cell motility. The prototypic structure of murine coronin I shows a seven-bladed β propeller stabilized by a C-terminal extension of the sequence. Coronins in yeast, C. elegans, Drosophila and mammals regulate actin functions in various ways. [Coronin 7 is exceptional because of its Golgi localization]. Coronins from several organisms have been reported to interact with the Arp2/3 complex and some to inhibit its activity. From electron tomograms it has been inferred that murine coronin I arrests the Arp2/3 complex in an inactive state through binding to its p35 subunit. Coronin localizes to Listeria comet tails and has been reported to facilitate the cofilin-mediated disassembly of actin along the tails. Accordingly, the localization of Dictyostelium coronin to sites of actin disassembly is most evident in actin tails that are formed at retracting phagosomes. While myoB and the Arp2/3 complex accumulate close to the membrane of the phagosome, coronin is recruited specifically to the decaying end of the tails. Finally, GFP-tagged clathrin light-chains are used in this study to mark sites of membrane recycling. Clathrin is known in fibroblasts and yeast to cooperate with both actin and the Arp2/3 complex in mediating endocytosis.

As Dictyostelium lives in a natural habitat of deciduous forest soil where its cells do not find specific extracellular matrix proteins such as fibronectin, the cells adhere to various surfaces by physical interactions. Nevertheless, specific membrane proteins are required for these “unspecific” interactions. Mutational analysis revealed that different proteins are responsible for binding to either hydrophilic or hydrophobic surfaces. Therefore, we have performed experiments on hydrophilic glass as well as alkane-coated hydrophobic surfaces. Since there were no principal differences in the behavior of cells on one or the other type of substrate, we argue that non-monotonic spreading is intrinsic to Dictyostelium cells and most likely other cells with highly dynamic actin-network organization.

Results

Discrimination of adhesive and non-adhesive surfaces by Dictyostelium cells. As a prelude to the image analysis of cell spreading in D. discoideum, we have incubated cells on a variety of adhesive and non-adhesive glass surfaces. The network of actin filaments associated with the substrate-attached cell membrane was visualized using GFP-LimEΔ as a marker for filamentous actin. TIRF microscopy proved to be the method of choice for visualizing dynamic actin networks in the cortex of Dictyostelium cells, and is used here to demonstrate the actin structures formed in spreading cells.

Figure 1 shows cells on three surfaces that allow the cells to spread (A–F) and on two surfaces from which the cells are repelled (G and H). The non-adhesive surfaces are contacted only by small areas of the cell body or by the tips of filopodia (Fig. 1G and H and Suppl. movie 1). The basic structure in spreading cells is an essentially two-dimensional network of actin filaments, or of small bundles of these filaments, beneath the plasma membrane (Fig. 1A and movie 2). Filopodia are rich in bundled actin filaments and therefore strongly labeled (Fig. 1D). Inserted into the network are dense fabrics of actin filaments known to be associated with the Arp2/3 complex. These assemblies of densely packed filaments may be localized to one or two protrusions of the cell (arrowheads in Fig. 1A and D), less often they are organized into propagating waves; one such wave in the form of an expanding wheel-shaped structure is shown in Figure 1B. Most abundant are short-lived actin patches of less than one micrometer in diameter (Fig. 1C and E). Figure 1F serves as a control showing a spread cell expressing free GFP. This protein is uniformly distributed in the cytoplasm, providing proof for the absence of unspecific binding of the fluorescent tag to any organelle or cytoskeletal structure.

In suspension, the cells of D. discoideum are irregular in shape and capable of forming filopodia and lamellipodia-like protrusions. Consequently, when these cells settle they touch the substrate with their protrusions or with the major, rounded portion of their body (movie 2). In this report we first focus on the fluctuating increases and decreases in the size of the contact area that are characteristic of spreading Dictyostelium cells, and subsequently explore re-organization of the actin system underlying these fluctuations.

Non-monotonic dynamics of cell spreading. To analyze the spreading dynamics of Dictyostelium cells in quantitative terms, we determined the size of the surface area in contact with a substrate as a function of time (Fig. 2). TIRF imaging guaranteed that fluorescent areas recognized in spreading cells are within a range
Figure 1. Cells of *D. discoideum* spreading on differently coated glass surfaces (A–F) and non-spreading cells on passivated surfaces (G and H). Structures in the cell cortex were visualized by TIRF microscopy. (A and B) cleaned glass; (C and D) glass coated with dichlorodimethylsilane; (E and F) coated with fluoralkane; (G and H) glass passivated with F-127 (G) or polyethyleneglycol, PEG 2000 (H). The actin network beneath the substrate-attached cell surface was labeled with GFP-LimEΔ, except for the control cell shown in (F), which expressed free GFP in the cytoplasm. Arrowheads in (A and D) indicate dense accumulation of actin in front regions of the cells. Exposure time 9 milliseconds. Bars, 5 μm.

Figure 2. Fluctuations in the area of cell-to-substrate contact on a non-adhesive (A), a hydrophilic (B), and a hydrophobic (C) surface. The time of first cell-substrate contact is set to zero seconds. (A) Two cells attempting to attach to a PEG 2000 coated glass surface. The cells adhered iteratively with small areas of their surface on the passivated glass but the adhesion was only temporary. (B) Fluctuations in contact area during cell spreading on cleaned glass surfaces. The contact areas of the five cells plotted indicate continued increases and decreases in area size over the entire 4 minutes of recording. (C) Fluctuations in contact area during cell spreading on fluoralkane. Three cells were recorded for up to 10 minutes in order to demonstrate that sizes of the contact area do not equilibrate after longer periods of time. Sizes of the contact area were determined on TIRF recordings of cells labeled with GFP-LimEΔ, using its distribution in the cytoplasm to identify the substrate area covered by a cell. The algorithm applied reveals the total contact area of a cell even if this area is dispersed. This means that filopodia or lamellipodia that touch the substrate remote from the compact cell body are included. Note differing scales on the ordinates. The insets in (A–C) zoom into the first 30 seconds of cell spreading.
of about 100 nm apposed to the reflecting glass surface. Using the low cytoplasmic background of the LimEΔ label to circumscribe the area of contact, we followed single cells from their first interaction with the substrate for periods of up to 10 minutes. As a non-adhesive substrate we have chosen PEG 2000, and as adhesive substrates cleaned glass and fluoralkane-coated glass. On the non-adhesive substrate, phases of attachment alternated in the 10–100 seconds range with phases of detachment. The small areas of contact did not expand upon these recurrent interactions, indicating that they are insufficient for a cell to spread (Fig. 2A).

On the hydrophilic surface of cleaned glass, to which the cells adhered, the size of the contact area acquired a first maximum within the first 10 seconds but never reached an equilibrium during the 5-minute periods of observation (Fig. 2B and inset). These fluctuations in cell-substrate interactions may be a characteristic of cell spreading in Dictyostelium cells, or they may be a specific way of interacting with a blank glass surface. To distinguish between these possibilities, we subjected cells to the most strongly hydrophobic surfaces employed, those coated with fluoralkane. Again, phases of increase in size of the contact area were separated by phases of decrease. Even during the first 10 seconds of spreading the area size did not increase in a monotonic manner (Fig. 2C and inset).

In conclusion, on both adhesive substrates the cells remain, after the first phase of attachment, permanently in contact with the substrate, but the contact area continuously increases and decreases in size. The question is how the gain and loss of contact area during the spreading process is linked to the local accumulation of actin-associated proteins that either promote protrusion or retraction. We addressed this question by investigating spreading in double-labeled cells, which enabled us to relate the localization of these proteins to sites of actin polymerization or depolymerization.

**Front-to-tail differentiation.** To monitor front and tail specification during spreading, the cells were labeled with mRFP-LimEΔ and GFP-mysin-II. In the first 10 seconds, a protruding front rich in polymerized actin is distinguishable from a retracting tail, where filamentous myosin-II is accumulating. The protruding fronts correspond to the actin-rich regions distinguished in Figure 1 from the actin network that surrounds the entire cell.

Figure 3 and movie 3 illustrate how polarity is established in a spreading cell by the sorting out of regions in the cell cortex that become either rich in actin or myosin-II. Figure 3A shows that the filaments of myosin-II are initially distributed over the entire visible area of the cell cortex. Subsequently, multiple actin-rich protrusions are formed, which in the cell shown are all but one short-lived. These protrusions are depleted of myosin-II. At the end of the sequence, the persisting front co-exists with a single myosin-II-rich region at the opposite end of the cell, which means that bipolarity is temporally established.

Iterative protrusion and retraction, caused by the interconversion of front and tail regions, is exemplified in Figure 3B. At the beginning of spreading, this cell forms three actin-rich protrusions from which large myosin-II filaments are excluded. Repopulation with myosin-II is most evident during retraction of the third front (compare the 66 and 105 second frames in Fig. 3B and view movie 3). This recording shows that the interplay of actin and myosin-II recruitment causes the spreading process to proceed in a non-monotonic manner: phases of expansion are interrupted by retraction. In the extreme case, the entire substrate-attached surface area is occupied by myosin-II filaments and actin-rich protrusions are totally missing (218 seconds frame). In summary, the data obtained with cells labeled for actin and myosin-II indicate that the gain and loss of contact area is based on the reprogramming of front to tail regions, and vice versa, in a spreading Dictyostelium cell.

**Fluctuating gain and loss of contact area.** Changes in the size of a contact area are the net effect of local gain, caused by protrusion, and local loss, caused by retraction. Hence a constant size of the contact area is equally consistent with a cell being quiet or being active in forming protrusions that are compensated by retraction. Likewise, increases and decreases in size of the contact are not necessarily reflected in translocation of the cell's geometric center. Therefore, we have separately analyzed gain and loss, the two determinants of area...
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Figure 4. Dynamics of protrusion and retraction in cells spreading on a fluoralkane-coated surface (A and B) or on a cleaned glass surface (C and D). Cells labeled with LimEΔ-GFP were recorded by TIRF microscopy at intervals of 1 second. (A and C) Sizes of the contact area are encoded in rainbow colors and with the velocity of translocation of the cells’ center of mass (black curves at the top). The local gain and loss was calculated by subtracting pixel-by-pixel and second-by-second the area in frame n from that in frame n + 10 (i.e., a frame recorded 10 seconds later). (B and D) Translocation of the cells’ geometric center with time is encoded in the same rainbow colors as for the sizes of contact area. The crossing point of the inserted lines refers to the starting position of the center of mass. Closed arrowheads indicate simultaneous protrusion and retraction, open arrowheads protrusion accompanied by retraction in phases of negligible translocation of the cells.

Myosin-IB and the Arp2/3 complex in spreading cells. Single-headed myosins have first been shown by Fukui et al.9 to localize to the front of Dictyostelium cells. The Arp2/3 complex constitutes the dense actin assemblies at the front regions of unstimulated and chemoattractant-stimulated cells.10,44 The participation of these proteins in reorganization of the actin system was studied in spreading cells by the labeling two-by-two of myoB, constituents of the Arp2/3 complex, and actin.

When GFP-myoB is combined with mRFP-LimEΔ, the two labels overlap at front regions, in patches, and in filopodia, although they do not completely coincide. The cell shown in Figure 5 contacts the substrate first through its filopodia. In the course of spreading, GFP-myoB as well as the actin label become concentrated at expanding zones of the cell border (38 seconds frame). This cell extensively blebs, a feature often but not always observed at the beginning of spreading. Initially, neither myoB nor actin is accumulated in the blebs (open arrowheads), but the rounded blebs become structured by the arrangement of these proteins in zonal patterns (closed arrowheads).

GFP-Arp3 is shown in Figure 6 to co-localize with the mRFP-LimEΔ label in two structures on the substrate-attached area of spreading cells: in expanding lamellipodia and in patches. In addition, this figure demonstrates that spreading can proceed by fusion of initially separate areas of contact with the substrate into a compact, fluctuating area of contact (149 to 236 second frames). Direct comparison of the localization of myoB and ARPC1, another member of the Arp2/3 complex, shows myoB to be strongly enriched in filopodia, relative to the enrichment of both myoB and the Arp2/3 label in lamellipodia (movie 5).

Coronin and the disassembly of actin. The repeated gain and loss of contact area suggests an alternation of actin polymerization and depolymerization that is correlated with local protrusion and retraction in a spreading cell. In order to visualize the dynamics of actin filament turnover we have used coronin, a protein shown to localize to sites of actin disassembly in the cells of Dictyostelium.11,12 In Figure 7, the red-green pattern depicts the enrichment of actin or coronin from the very beginning of spreading up to the establishment of a front-to-tail polarity. Initially, zones of actin enrichment are adjacent with coronin-enriched zones (open arrowheads in the 11 seconds frame). Subsequently, actin-rich front regions are linked at their base to a coronin-enriched layer (open arrowheads in the 15 seconds frame). A sequence of macropinocytosis, that happens to be...
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in the plane of focus, exhibits a comparable arrangement of actin and coronin (closed arrowheads). During progression of the cup, actin is localized toward its lumen and coronin toward the cytoplasmic face (46 seconds frame). During regression of the cup, coronin is dominating in the entire structure (53 and 62 second frames).

A clear case of switching from actin to coronin in the labeled area of a cell is provided by the reversal of cell polarity shown in Figure 8A. The cell spreads first by moving toward the bottom of the frame, with a band labeled red for actin at the very front, followed by a band labeled green for coronin (12 and 27 second frames). When the cell turns toward the top, the new front shows the same pattern while the previous front, now converted into a tail, becomes green (36 and 41 second frames). The cell shown in Figure 8B and movie 6 is of interest for two reasons. First, alternating protrusions and retractions of its leading edge are closely reflected in the local off

and on of coronin recruitment. Secondly, the cell shows numerous actin patches that are transiently formed at the substrate-attached cell surface. Coronin marks these patches in the phase of their disappearance (arrowheads in Fig. 8B and movie 6). In the following we characterize these patches in terms of the associated proteins and the sequence of their recruitment.

Clathrin-associated actin patches in spreading cells. Actin patches of less than one micrometer in diameter are regularly found at the substrate-attached area of migrating cells. Clathrin-coated structures are distributed over the substrate-attached surface of Dictyostelium cells with the exception of leading edges and filopodia (Fig. 9, see the 25 and 93 second frames). At any time, most of these clathrin-containing structures are mobile and free of actin. If one of these patches associates with actin (becoming yellow in the superimposed images), it stops movement and will soon disappear from the membrane, suggesting that an endocytic vesicle has been budded off (Fig. 9, arrowheads in the 16 to 25 and the 131 to 156 second frames, and movie 7). Classification of the actin patches revealed that more than 80 percent of them are engaged with clathrin. Similar numbers of coincidence were obtained for actin with coronin, Arp2/3, and myoB (Table 1). According to these data only a minority of actin patches, if any, are likely to be adhesion sites.

The temporal relationships between proteins associated with actin patches were quantified by plotting fluorescence intensities in the
patch area relative to the actin peaks (Fig. 10). Clathrin disappears from the membrane when actin is beginning to rise. The peaks of myoB and the Arp2/3 complex are not significantly shifted relative to the actin peak. Only the peak of coronin is postponed by 3 seconds, such that coronin recruitment reaches its maximum when actin sharply declines. This delay in the engagement of coronin is in accord with the color shift in the patches shown in Figure 8B.

**Discussion**

In this study we have related iterative shape changes in spreading cells of Dictyostelium to the re-organization of their actin system on the scale of seconds. The cells were double-labeled with fluorescent proteins in order to visualize the actin network in the cell cortex together with proteins responsible for its function and regulation. The principal result is that these highly motile cells never reach an equilibrium size of the contact area. Independent of whether the surface is hydrophilic or hydrophobic, sizes of the contact area may attain maxima at any time during the migration of a cell on a substrate surface (Figs. 2 and 4).

Mouse embryonic fibroblasts have served as a standard model for the spreading of cells by lamellipod expansion on an adhesive surface. Under serum-free conditions, these cells have the advantage of being unpolarized and thus to spread with a circular boundary, which facilitates the measurement of spreading. After the cells have passed three phases distinguished by their spreading dynamics, they will finally attain a constant size of the contact area.

The spreading of HeLa cells on a fibronectin-coated surface is characterized by a scaling law describing the radius R of the contact area to increase with $R \propto t^{1/2}$. During this increase, the actin network in the cell cortex is assumed to behave as a viscous shell enclosing a liquid interior of the cell. Only in a subsequent step, the viscoelastic behavior of spreading cells is found to be blurred by protrusions formed on the basis of actin polymerization. Hence, in fibroblasts as well as HeLa cells the spreading period is separated by its characteristic dynamics from the subsequent migration of cells on the substrate.
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Time is indicated in seconds after first contact of the cell with the substrate. Exposure time of 50 milliseconds for GFP and 100 milliseconds for mRFP.

Figure 9. Clathrin-associated actin dynamics in a spreading cell. This cell expressing GFP-clathrin light chain (green) together with mRFP-LimEΔ (red) shows clathrin-dependent activities throughout spreading. Beginning with the first contact, clathrin-coated structures are present on the substrate-attached membrane area. Arrowheads point to the sporadic association of these structures with actin (color change from green to yellow) followed by their disappearance from the plane of focus. Clathrin-coated structures are excluded from the dense actin assemblies at leading edges and also from filopodia. Cells spreading on a fluoralkane surface were recorded by spinning disc microscopy with focus on the substrate-attached surface and an exposure time of 50 milliseconds for GFP and 100 milliseconds for mRFP. Time is indicated in seconds after first contact of the cell with the substrate. Bar, 5 μm.

Figure 10. Recruitment of clathrin and actin-binding proteins to actin patches. Cells double-labeled with mRFP-LimEΔ for actin and with one of four GFP-tagged proteins were allowed to spread on fluoralkane surfaces. Cells were imaged by TIRF microscopy, and fluorescence intensities determined in a circular area of 1.4 μm diameter centered on stationary actin patches. The time of maximal intensity in the actin label [Lim] is set to zero on the time axis, and temporal changes in fluorescence intensities of the four other proteins are related to this peak: CLC, clathrin light chain; MyoB, myosin-IB; Arp3, subunit labeling the Arp2/3 complex; Cor, coronin. Fluorescence intensities before occurrence of the patches were set to zero and peak intensities were normalized to 1.

myosin-IB redistribution reflects the depolymerization of three threonine residues in the tail region of the heavy chains. Therefore, myosin-II redistribution reflects the spatiotemporal control of actin assembly and disassembly. The pattern of actin and coronin in spreading cells reflects the spatiotemporal control of actin assembly and disassembly. The localization of coronin only 2–3 μm behind the zones of actin polymerization underscores the notion that not only assembly but also the disassembly of actin accompanies the global spreading of a cell (Fig. 8B). In fact, it takes less than 10 seconds for a small protrusion built by actin polymerization to become disassembled, and less than 30 seconds for a lamellipod to retract.

The capability of the actin system to participate at the same time in different activities is demonstrated by the dynamics of

### Table 1 Coincidence of GFP-labeled proteins with actin patches on the substrate-attached surface of spreading cells

| GFP-labeled protein | Total count | Coincident patches | Percentage of coincidence |
|---------------------|-------------|---------------------|--------------------------|
| Clathrin            | 161         | 132                 | 82%                      |
| Coronin             | 88          | 83                  | 95%                      |
| Arp3                | 157         | 149                 | 95%                      |
| MyoB                | 151         | 134                 | 90%                      |

GFP-labeled protein: for clathrin, the light chains were labeled. Total count: number of actin patches examined for coincidence with the respective GFP-labeled protein.
clathrin-coated structures in spreading cells. While the contact area is expanding, clathrin-coated pits acquire clusters of polymerized actin and disappear from the substrate-attached cell surface. This activity suggests that, even when the contact area expands, membrane is locally removed by internalization (Fig. 9). The same proteins as associated with the front regions of spreading cells are recruited to the clathrin-associated actin patches. The Arp2/3 recruitment is consistent with data on clathrin-dependent endocytosis in fibroblasts and yeast. Coincidence of myoB recruitment with the peak of actin indicates the participation of this motor protein in clathrin-dependent membrane dynamics. A delay of the coronin peak by 3 seconds relative to the actin peak confirms that coronin is specifically recruited to sites of actin disassembly.

The finding that in spreading cells of Dictyostelium the contact area never stays constant is consistent with previously reported fluctuations in the interaction of migrating Dictyostelium cells with a substrate surface. The fluctuations were most pronounced on mica, a weakly adhesive substrate, indicating that they are autonomously generated in the cells and suppressed rather than enhanced by interaction of the cells with the substrate. We conclude that the spreading of Dictyostelium cells, as well as their migration, is dominated by autonomous changes in activities of the actin system. These changes are brought about by the local and short-lived recruitment of proteins from the cytosol to the cell cortex. The interplay of these proteins generates the fluctuating pattern of protrusions and contractions that govern the spreading process in Dictyostelium cells.

Materials and Methods

Cell strains and cultivation. Cells of the Dictyostelium discoideum strain AX2-214 were transformed by electroporation with integrating vectors encoding LimEΔ-GFP, or LimE tagged with the Mars version of mRFP, and one of these GFP-fusion proteins under control of the actin-15 promoter: GFP-Arp3Δ mRFPΔ-ARPC1 (ArpD, p41-Arc), coronin-GFP, GFP-myoB, GFP- myosin-II, or GFP-clathrin light chain.

The cells were cultivated at 23 ± 2°C in petri dishes with nutrient medium containing maltose supplemented with appropriate selective agents (G418 and/or blasticidin) for maintenance of the expression vectors. Prior to imaging, cells from non-confluent cultures were washed twice in 17 mM K/Na-phosphate buffer (PB), pH 6.0, suspended and transferred onto the respective surface.

Treatment of glass surfaces. Cleaned glass surfaces. Glass cover slips were cleaned by immersion in the following solutions: 55% nitric acid, 3:1, three 1-minute water rinses before drying in nitrogen. Following 1 hour of silanization in 0.05% dichlorodimethylsilane in trichloroethylene, cover slips were washed four times in methanol while sonicated. After three further rinses with nano-pure water, the cover slips were stored dry.

Passivation with F-127. Cover slips coated with dichlorodimethylsilane were covered for 5 minutes with 1% Pluronic F-127 Block Copolymer surfactant solution (Sigma) and extensively rinsed with PB.

Passivation with PEG 2000. Plasma activated cover slips were immersed in 250 μM mPEG 2000-silane derivate in dry toluene with catalytic amounts of triethylamine, and heated for 16 hours at 80°C. After rinsing with ethyl acetate and methanol, the samples were blown dry with nitrogen, stored under an argon atmosphere and used within two days.

Live-cell imaging. TIRF microscopy. Spreading cells were illuminated through an alpha-Plan-Fluar 100x/1.4 NA oil immersion objective on a Zeiss Axiovert 200 M microscope equipped with a 1.6x optovar. For single-emission TIRF microscopy, we used a single line, single mode 488 nm Argon laser (Laser Technologies Inc., Batavia, IL 60510, USA) with 10–15 mW output power and a TIRF GFP filter set with a band pass emission filter (Z488/10X, Chroma Technology, Rockingham, VT 05101, USA). TIRF conditions were adjusted using a home made TIRF condenser. An Ixon 14 bit back illuminated frame transfer camera (DV837/DCS-BV, 512 x 512 pixels, pixel size 16 x 16 μm; Andor Technology, Belfast BT12 7AE, Northern Ireland) controlled by SOIliS software (Andor Technology) was used for short exposure times on the order of 10 milliseconds.

For dual-emission TIRF microscopy, the cells were illuminated with the 488-nm line of a Coherent Innova 90 Argon/Krypton ion laser (Coherent, Santa Clara, CA 95054, USA), coupled through a single-mode fiber (OZ Optics, Ottawa, Ontario KOA ILO, Canada) to a TIRF condenser (Till-Photronics, D-82166 Gräfelfing, Germany). A TIRF GFP filter set used for excitation (Z488/10X, Chroma Technology, Rockingham, VT 05101, USA) was combined on the emission side with a Hamamatsu W-view mirror design (Hamamatsu Photonics Deutschland, D-82211 Herrsching, Germany). Filters in the W-view were arranged such that the stronger GFP emission went through the upper light path containing lenses to correct for zoom and focus differences, and the weaker mRFP emission went through the lower light path without further lenses (first dichroic, 590SP; second dichroic, 590LP; both from Chroma Technology, Rockingham, VT 05101, USA). The channels were efficiently separated by a 520/35 lower emission filter for GFP and a 624/40 upper emission filter for mRFP (both from Semrock, Rochester, NY 14624, USA). Images from a Roper 16 bit back illuminated frame transfer camera (MicroMax 512BFT CCD, 512 x 512 pixels, pixel size 13 x 13 μm; Princeton Instruments, NJ 08619, USA) were acquired by MetaMorph software (Universal Imaging, Visitron Systems, D-82178 Puchheim, Germany). Mirrors of the W-view were adjusted to ensure exact alignment of the GFP and mRFP images on the respective halves of the camera chip using an x-y scanner calibration slide (Carl Zeiss MicroImaging, D-07745 Jena, Germany) and a tetra Speck bead slide (Molecular Probes/Invitrogen). Laser output power was adjusted to 50 mW to obtain an optimal image-to-noise ratio at minimal impact on the cells. Camera exposure times of 10–100 milliseconds proved to be optimal.
Spinning disc confocal microscopy (SDCM). Time-lapse sequences were acquired using an Ultra View ERS-FRET System (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA 02451, USA) on a Nikon TE-2000 microscope equipped with a Plan-Apochromat VC 100x, 1.4 NA oil objective (Nikon GmbH, D-40472 Düsseldorf, Germany). GFP and mRFP were excited sequentially with the 488- and 568-nm line, respectively. Emission was detected through a dichroic mirror and double band-pass emission filter on an EM-CCD camera.

Image analysis. Cell identification and tracking. We determined the contact area of fluorescent cells in TIRF images using custom made software employing the density-based clustering algorithm DBCSCAN51 in combination with a threshold setting that separates pixels with high fluorescence intensity from the background outside of a cell. Dense colocalizations of the high-intensity pixels are combined to individual clusters. Separate clusters including attachment areas of lamellipodia and filopodia were manually united with the main area of the cell body. The sum of these clusters represents the contact area of a cell.

For the tracking of motile cells in a time series, the center of mass of all clusters attributed to a cell was calculated by averaging over the coordinates of all its pixels. In Figure 4B and D the movement of the center of mass is plotted over time.

Gain and loss of contact area. Increases and decreases of contact area were calculated by comparing each frame of a time series with a frame 10 seconds before. In Figure 4A and C, new area is plotted in red and lost area in green. Similarly, in the supplementary movie five frame 10 seconds before. In Figure 4A and C, new area is plotted in red and lost area in green. Similarly, in the supplementary movie five frames with high fluorescence intensity within the mask was extracted using an ImageJ plug-in described by Bretschneider et al.7 In the simultaneous recording of a GFP-labeled protein, fluorescence intensities were extracted using the mask in the corresponding neously recorded channel of a GFP-labeled protein, fluorescence ImageJ plug-in described by Bretschneider et al.7

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