Local force induced conical protrusions of phagocytic cells

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Summary

Magnetic tweezers were used to study the passive and active response of macrophages to local centripetal nanonewton forces on β1 integrins. Superparamagnetic beads coated with the β1-integrin-binding protein invasin were attached to J774 murine macrophages to mimic phagocytosis of bacterial pathogens. Forces exceeding ~0.5 nN induce the active formation of trumpet-like protrusions resembling pseudopodia after an initial elastic deflection and a response time of ~30 seconds. The speed of advancement of the protrusion is $\langle v \rangle = 0.065 \pm 0.020$ μm second^{-1} and is force independent. After saturation (after about 100 seconds) the protrusion stops abruptly and is completely retracted again against forces exceeding 5 nN with an effective relaxation time of ~30 seconds. The active protrusion is tentatively attributed to the growth of the actin cortex in the direction of the force, and evidence for the involvement of actin is provided by the finding that Latrunculin A abolishes the activated cone growth. The growth is assumed to be activated by cell signaling mediated by the invasin-specific integrins (exhibiting β1 chains) and could play a role in phagocytic and protrusive events during immune response by macrophages.

Key words: Magnetic tweezers, Phagocytosis, Yersinia, Invasin, Macrophage, J774, Protrusion

Introduction

Cells are able to convert mechanical stresses into intracellular signals through integrin receptors (Choquet et al., 1997; Ingber, 1991; Sheetz et al., 1998; Wang et al., 1993) and stretch-activated cation channels (Glogauer et al., 1997; Glogauer et al., 1995; Lee et al., 1999; Sokabe et al., 1997), which in turn regulate many biological functions of the cell, ranging from migration to differentiation (for a review, see Chicurel et al., 1998). The activation and reorganization of the cytoskeleton is a ubiquitous physiological response of the cell to a mechanical stress. A typical consequence of external forces can be for example an increase in the cortical tension, which enables the cell to resist compression (Glogauer et al., 1998). When applied locally with magnetic tweezers, this force can stimulate the cytoskeletal activity and lead to a directed displacement of the cell (Toyoizumi and Takeuchi, 1995).

Reorganizations of the actin-based cytoskeleton evoked by mechanical forces and other external stimulations (e.g. adhesion) are often difficult to distinguish. Micromechanical measurements of forces or of changes in the viscoelastic moduli associated with the reorganization of the cytoskeleton provide a valuable tool to quantify such changes.

An example of the cytoskeletal reorganization induced by adhesion is the attraction and engulfment of pathogens by macrophages during phagocytosis. Micromechanical studies of this process were performed by the micropipette aspiration technique (Evans et al., 1993; Swanson et al., 1999; Zhelev and Hochmuth, 1995). In the experiments by Evans et al., for instance, the force generation associated with the engulfment of pathogenic yeast cell by macrophages was studied. It was found that if the neutrophils are aspired into the micropipette with fixed lengths of protrusion, the opposite end of the cell first spreads over the pathogen at constant tension. It then suddenly stops and starts to build up a cortical tension, suggesting that the spreading and the force generation are subsequent processes. To gain insight into these sequences of steps during phagocytosis from a different perspective we studied the response of J774 macrophages to local centripetal pulling forces applied by magnetic tweezers (which allowed us to generate forces of up to 5 nN).

The magnetic beads were coated with invasin, a surface protein of pathogenic Yersinia species, which causes gastrointestinal infection by invasin-triggered invasion into the intestine. This protein binds to integrins containing β1 chains and induces the recruitment of actin microfilaments and actin-associated proteins, such as filamin and talin. This cytoskeletal reorganization is also assumed to be the first step during the process of internalization of pathogens. In the present study the external force on the invasin-coated bead is applied immediately before the spreading of the phagocyte over the bead starts, and it is oriented in the same direction. Such experiments are expected to yield insight into the time evolution of the formation of cellular protrusions at the initial state of phagocytosis and into the process of generation of retraction forces resulting in the engulfment of the particles. These forces are important if phagocytes have to remove pathogens adhering strongly to surfaces, for example, to other cells or tissues. One advantage of the magnetic tweezers

technique is that complex force scenarios can be applied such as sequences of pulses or staircase-like force programs. We show that it is possible to distinguish between active and passive responses of the cells to external forces by analyzing the change in deflection of the cell in response to small changes in force.

Materials and Methods

The magnetic tweezers setup

The experimental setup of the microrheometer has been described previously (Bausch et al., 2001; Ziemann et al., 1994). The magnetic field is generated by a copper solenoid with a soft iron core exhibiting a sharp edge protruding from the solenoid. The tip of the core penetrates the measuring chamber in such a way that the distance between the tip and the magnetic bead can be adjusted down to a minimum value of 10 μm. The measuring chamber consists of a copper block into which a teflon frame (24×24 mm) is fitted, which serves as holder for the glass plates on which the cells are deposited. The volume of the measuring chamber is about 100 μl. The whole setup (the chamber and the magnetic coil) can be fixed on the microscope stage. The current entering the solenoid is generated by a voltage-controlled current supply. It transforms the voltage signal of a function generator FG 9000 (ELV, Leer, Germany) into a current signal exhibiting amplitudes of up to 4 A. Depending on the distance between the bead and the coil, forces in the order of 10 nanonewtons can be generated.

Sample preparation

The experiments were performed with J774 mouse macrophages (European Collection of Animal Cell Cultures, Salisbury, UK). Cells were cultivated in an incubator at 37°C and 5% CO_2. The cell culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies GmbH, Eggenstein, Germany).

Tosyl-activated superparamagnetic Dynabeads (Dynal, Hamburg, Germany) with a diameter of 4.5 μm were covered with invasin proteins according to the procedure provided by the supplier. Expression and purification of this protein is described in a previous paper (Wiedemann et al., 2001). In order to verify the binding of invasin, the beads were incubated at 4°C with rabbit anti-invasin antibody, diluted 1:1000. Beads were then washed three time with 1% PBS to remove unbound antibodies and then incubated with 488-labeled goat anti-rabbit antibodies, diluted 1:200 (Molecular Probes). Beads that are effectively covered by invasin exhibit a fluorescent rim under a confocal fluorescent microscope.

In order to define the initial conditions of the experiment we proceeded as follows. For each experiment the cells were detached from the culture dish and transferred onto coverglasses. After incubating with cell culture medium for 24 hours, a coverglass covered with cells was mounted on the copper block of the measuring chamber. Invasin-coated beads were then carefully added to the chamber, on top of the J774 macrophages. Immediately after this step, the motion of the beads towards the vicinity of the cells was followed by visual inspection. Occasionally, randomly distributed beads on the surface of the substrate are contacted by filopodia extending several μm into the medium and are attracted towards the cell body (L.V, unpublished). We selected beads attached to the cell, which were localized between the macrophage and the tip of the iron core. Thus, the external forces could be applied in the direction opposite to the active force exerted by the cell to engulf the bead. The distance between the cell and the tip varied between 20 and 160 μm, resulting in force amplitudes ranging from 0.1 to 9 nN. If no external forces are applied on the cell the bead is internalized after attachment to the cell within about 10 to 15 minutes.

To check the role of the actin cortex in our measurements we completely and rapidly exchanged the medium in a series of experiments by different stock solutions of medium containing Latrunculin A (Molecular probes) and dimethylsulfoxide (DMSO) (Molecular Probes), just before the engulfment of the bead. The concentration of Latrunculin A was varied from 1 μM to 10 μM; the medium:DMSO ratio was 1:150 to 1:1500.

Data acquisition

Both the position of the bead and the shape of the cell were observed with an AXIOVERT 100 phase contrast microscope (Zeiss, Oberkochen, Germany) using a ×32/0.75 objective. Images were recorded with a CCD camera connected to a numerical data acquisition system. The beads were tracked using homemade software with an accuracy of 10 nm (not available on the market).

Results

Fig. 1 shows the displacement over time of a bead subjected to a force pulse of 2 nN amplitude and 90 seconds duration in the direction perpendicular to the cell surface. Fig. 1A,B shows phase contrast micrographs at the beginning and the end of the force pulse. The deflection versus time curves exhibit typical features of creep response curves of viscoelastic bodies exhibiting plasticity. It shows firstly an instantaneous elastic response with an amplitude of about $\Delta x \sim 0.3 \pm 0.1$ μm, secondly a slowing down of the extension and thirdly a well defined linear flow regime.

The following experiments and considerations show that in the linear regime of deformation, the cell responds to the external force in an interactive way. The initial fast and passive response, which is also clearly reflected by the initial fast relaxation after switching off the force (see double arrow in Fig. 1), may be caused by two processes: firstly, an elastic deformation of the cell envelope leading to the bulging of the cell membrane in the direction of the force while the cell body remain fixed to the substrate; and secondly, a shear deformation of the whole cell body, resulting in a small displacement of the contour of the cell on the side opposite to the force direction (Lo et al., 1998). At present we can not clearly distinguish between these two alternatives. However, evidence for the second explanation is provided by the frequent finding that the rim of the cell opposite to the side of force application is displaced during the initial phase of the response. The slow cell deformation (characterized by the deflection of the bead with constant speed) can be described as an active growth of a cone-like protrusion. When the applied force is switched-off (before the active retraction sets in), the cell recovers its initial shape through the complete retraction of the protrusion. The process of phagocytosis of the bead will then continue until the bead is completely engulfed. At zero force the bead is rapidly internalized by phagocytosis.

In a second series of experiments we studied the force dependence of the growth rate of the cone for the same cell. For that purpose we induced the repeated formation of protrusions by application of a sequence of force pulses of different force amplitude. Before each new pulse we waited until the protrusions were fully relaxed. The different speeds of protrusion obtained with different forces are summarized in Fig. 2 for three cells. The numbers in the figures correspond to the number of the force pulse of each sequence. No correlation between the number of the pulse and the measured speed is

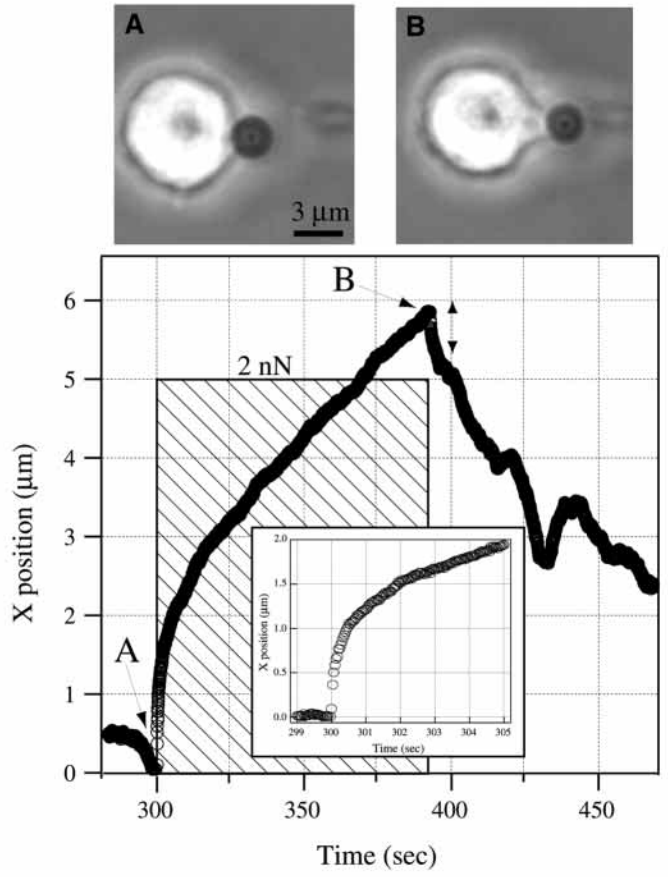

Fig. 1. Time evolution of formation of protrusion of macrophage induced by application of an external force pulse with an amplitude of 2 nN and 90 seconds duration. The phase contrast images (A,B) correspond to the beginning and the end of the pulse. The position of the magnetic tweezers in the direction of the force is plotted as a function of time. The inset shows the initial state of the deformation at higher time resolution to expose the initial elastic response more clearly.

found, which demonstrates that the history of the cell does not influence the measured growth speed. For forces larger than about 0.5 nN, the average speed varies between v=0.020 µm second^{-1} and 0.175 µm second^{-1} but no systematic force dependence is observed. The average value of the velocity obtained by evaluating 15 cells (49 measurements) is $\langle v \rangle = 0.065 \pm 0.020$ µm second^{-1}.

Further evidence for the active response of the cell is provided in Fig. 3. After application of a force of 2.55 nN for about 150 seconds the amplitude was reduced in a step-wise manner first to 1.7 nN, then to 0.85 nN and finally to 0 nN. Complete relaxation is observed only after switching off the force completely. By contrast, the step-wise reduction of the force to 1.7 nN and to 0.85 nN, respectively, leads first to a transient viscoelastic relaxation (indicated by arrows in the figure), but this process is then followed by a renewed growth of the cone-like tip with about the same velocity as before the reduction of the force. This type of behavior has been observed many times. It is interesting to note that the relaxation time of the transient retraction of the protrusion after the reduction of the force is of the same order (~20 seconds) as the response

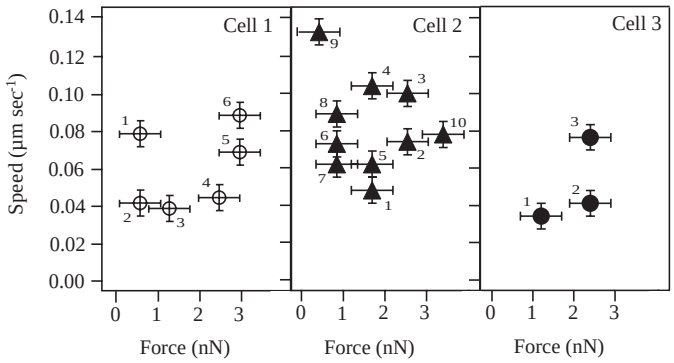

Fig. 2. Measurement of average velocity of beads pulled by the external force as a function of force amplitude. The enforced formation of protrusions was repeated several times for each cell. Examples are given for three different cells. On each image we have indicated the sequence number of the applied pulses in the whole series to demonstrate that the force does not depend on the prehistory of the sequence of the force application.

time characterizing the transition from the elastic deflection to the linear flow regime when the force is switched on.

After prolonged application of the external force, the cells retract the protrusions again against the largest external forces available (5 nN). An example is show in Fig. 4 for an applied force of 3 nN. It is seen that after application of the force for about 60 seconds the growth rate of the protrusion first decreases and the protrusion is finally reversed. Until now we had not found a systematic correlation between the onset of the retraction force and the length of the protrusion or the time over which the force is applied.

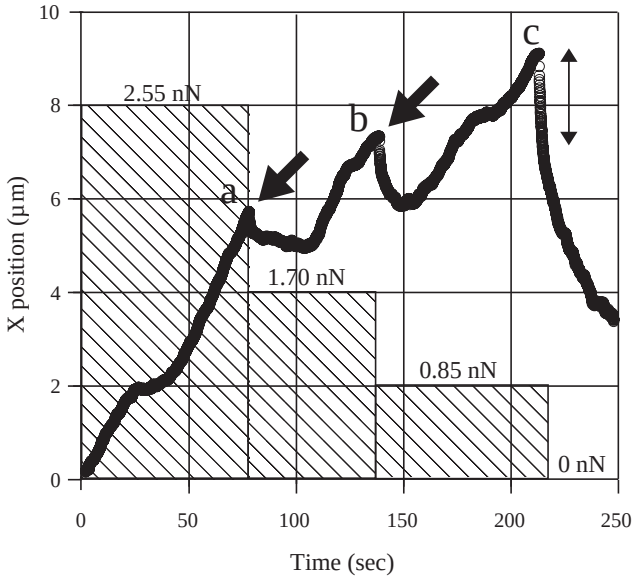

Fig. 3. Time evolution of deflection of the bead after stepwise reduction of the force amplitude from f_0=2.55 nN to 1.70 nN at position (a), to 0.85 nN at position (b) and to 0 nN at position (c). The arrows indicate the initial elastic relaxation after the reduction of the pulse. The double arrows at the end of the force pulse indicate the elastic regime of retraction.

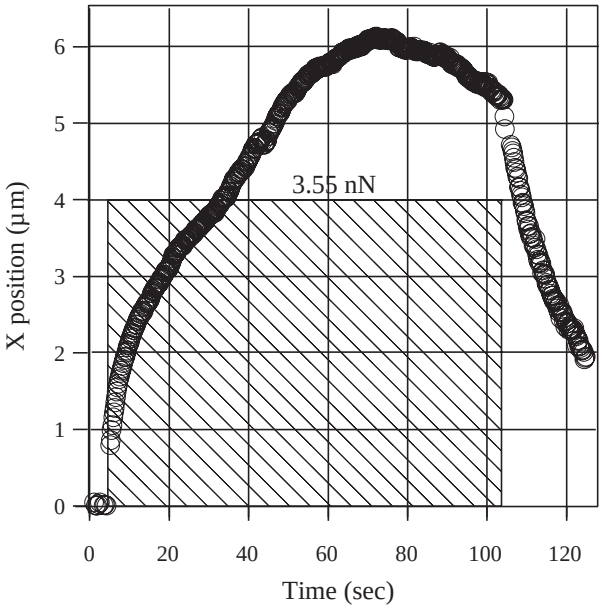

Fig. 4. Demonstration of active retraction of a protrusion against an external force of 3.55 nN.

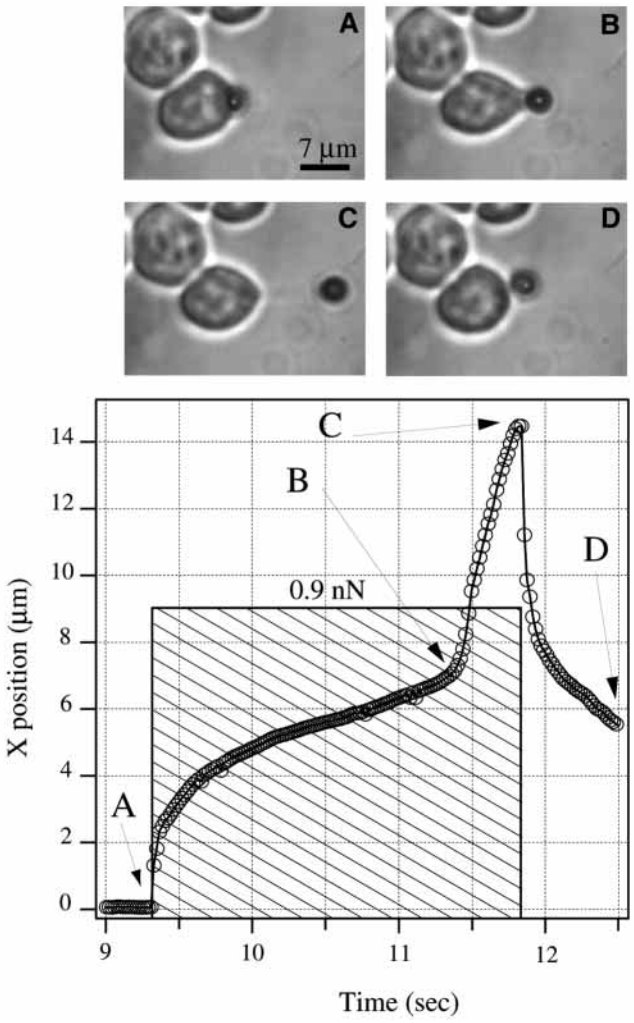

Fig. 5. Time evolution of protrusion of a macrophage after incubation with 10 μM Latrunculin A for 4 minutes. Phase contrast images at the beginning of the pulse (A), at the end of the viscoelastic response (B), at the end of the force pulse (C) and after relaxation (D) are shown. Note that the flow velocity in the viscoelastic regime (v~1.6 μm second^{-1}) is much higher then in the case of control cells.

To clarify the role of the actin network in the active growth of the cone we studied the response of cells treated with Latrunculin A. This agent strongly binds to monomeric actin and thus prevents the generation of a new actin cortex (Spector et al., 1983). We found two scenarios. In many cases (such as shown in Fig. 5) a viscoelastic response is found, consisting of an elastic deflection, a relaxation process and a flow regime (A to B in Fig. 5), which is followed by the pulling of a tether (B to C in Fig. 5). In some cases the viscoelastic response is also observed but is preceded by the formation of a short tether. In the first scenario the viscoelastic response and flow regime (A to B in Fig. 5) is also associated with the formation of a cone-like protrusion. However, this is a passive bending deformation since the flow rate is 1.6 μm second^{-1} and is thus much faster than the active process (<v>=0.065±0.020 μm second^{-1}).

Discussion

One purpose of the present work was to explore the potential application of the magnetic tweezers technique to study active viscoelastic responses of cells to local mechanical forces and to gain insight into local force-induced reorganizations of the actin cortex. A general problem of micromechanical studies of cells is to distinguish between passive viscoelastic responses and active processes mediated by the mechanical forces. With the magnetic bead microrheometry it is possible to distinguish between these processes by analyzing modification of the elastic response induced by small step-like changes of the extrinsic force (as shown in the example of Fig. 3).

The mechanical response of the macrophage to local force pulses applied on invasin functionalized beads, which are bound to integrins exhibiting β1 chains, is characterized by the following features:

(1) The response consists of three regimes: a fast elastic deflection, a slowing down of the deflection and a linear flow regime. The linear regime is observed above a threshold force of the order of 0.5 nN and the speed of deflection does not depend systematically on the amplitude of the applied force (Fig. 2).

(2) A reduction in the force by small steps within the linear flow regime leads to a transient relaxation of the deflection, which is however followed by a renewed advancement of the tip with the same speed (Fig. 3). The transient relaxation time agrees roughly with the time of transition between the elastic and the flow-like regime of deflection and is about 30±5 seconds.

(3) If the force is switched off completely, the protrusion relaxes to its original shape and the process consists of a fast elastic regime followed by an approximately exponential relaxation process. The relaxation time (~20 seconds) is of the same order of magnitude as the response time characterizing

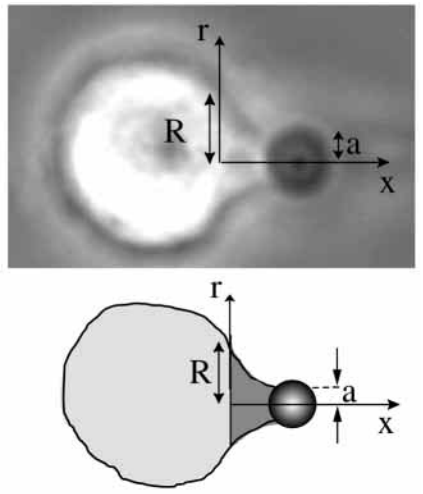

Fig. 6. Contour of trumpet-like protrusion of a cell enforced by nearly point-like force applied to the tip of the protrusion. R is the radius of the disk and a the radius of the bead.

the transition from the elastic deflection to the linear flow regime.

(4) In several cases long protrusions are retracted against the external force even at amplitudes of 5 nN (Fig. 4). Again the speed of retraction does not change if the force is reduced in a step-wise manner (with the exception of a small elastic deflection), showing again that the retraction is an active process.

(5) Latrunculin A impedes the interactive formation of a growing cone.

The threshold behavior of the cone growth process is similar to that of the tension-induced elongation of axons by forces applied through microglass needles to the tip of the growth cones of neurons (Zheng et al., 1991). The threshold force in this case varied between 0.5 and 1.5 nN and is thus similar to the present value of ~0.5 nN. Threshold behavior has also been reported for the formation of tube-like protrusions by aspiration of the cell envelope by micropipettes (Merkel et al., 2000; Zhelev and Hochmuth, 1995). However, in these experiments the threshold tension is an order of magnitude higher (typically 2 nN) and is determined by the fracture of the bilayer membrane from the actin-based cytoskeleton.

The force-induced elongation of the protrusion of macrophages and of the axon of neurons (Zheng et al., 1991) differ in one aspect. The growth rate of neurites is proportional to the applied stress, whereas no correlation between rate of advancement and the applied stress was observed in our case. The average speed of advancements of the neurite at 2 nN is a factor of three smaller than the average value of $<v>=0.065$ μm second^{-1} observed by us.

There are some interesting similarities between the force-induced growth of the cone-like protrusion and the spreading of the envelope of macrophages over pathogenic cells (Evans et al., 1993), although the driving force in this case is cell-cell adhesion. First, the speed of advancement of the cell lobe over the pathogen ($v\sim0.1$ μm second^{-1}) is similar to the cone growth rate ($<v>\sim0.065$ μm second^{-1}). Secondly, the advancement of the cell lobe over the pathogenic cell stops abruptly (after about 30 seconds) and a retraction force builds up, which is similar to the behavior of the macrophage shown in Fig. 4. The retraction force exhibits a value of about 10 nN and is thus beyond the force range of our technique.

Several observations of our study can be explained by assuming that the force-induced cone formation is associated with the formation of an actin cortex. One source of evidence comes from the experiment with Latrunculin A. The rather fast viscoelastic response associated with the formation of a conical deformation (Fig. 6A) and the subsequent tether formation is attributed to the partial decoupling of the actin cortex from the plasma membrane and the impediment of actin repolymerization by this agent. The decoupling facilitates tether formation owing to the fracture of bonds between the actin cortex and the intracellular domains of membrane receptors such as integrins (Hochmuth et al., 1996).

The sequestering of G actin by Latrunculin is expected to prevent the repairing of the fractures. The speed of the cone growth agrees reasonably well with biochemically stimulated growth rates of actin networks, such as the comet-like tails of listeria bacteria ($v\sim0.1$ μm second^{-1}) (Noireaux et al., 2000). These tails grow by assembly of a new actin gel at the surface of the bacteria through a mechanism similar to the formation of lamellipodia; that is by polymerization of actin between the advancing membrane lobe and the actin cortex by new synthesis of an actin network beneath the advancing membrane lobe (Borisy and Svitkina, 2000).

The protrusion assumes a conical shape, exhibiting a smooth transition into the main cell body (Fig. 6). The situation is very similar to the generation of a trumpet-like protrusion generated by a local force in the center of a circular disk of radius R. Following Bruinsma (Bruinsma, 1996), the contour of such protrusions is approximately given by $X(r)=X(a).\ln(R/r)/\ln(R/a)$, where a is the radius of the contact area between the bead and the cell membrane and R is the radius of the disk, which is about equal to the radius of the cell. For our case ($a\approx2$ μm and $R\approx10$ μm) the above equation holds with an accuracy of 20%. The extension Δx is proportional to the force f_0 ($f_0=\Lambda.\Delta x$). The force constant is $\Lambda=2\pi\gamma/\ln(R/a)$. This shows that, to a first approximation, the cortical tension does not depend on the length of the protrusion. It provides the driving force for the retraction of the protrusion after switching off the force before the active contraction sets in. The close analogy between the observed cellular deformation and the trumpet-like protrusions generated in a circular plate through a local force strongly suggest that the constant tension is built up within a thin layer close to the cell envelope.

The constant speed of advancement $<v>$ of the tip could be explained by two mechanisms:

First it could be determined by the rate of formation of free volume at the advancing tip generated by the force-induced disruption of the lipid/protein bilayer from the cytoskeleton. If this disruption is the rate-limiting step, the process corresponds to the Brownian ratchet model of cell locomotion (Mogilner and Oster, 1996). This model contradicts our finding of a much lower threshold force (~0.5 nN) than the fracture force required for the disruption of the actin-membrane coupling (Merkel et al., 2000). A second possibility is that the point force mediates the activation of a cell signaling pathway that controls the growth of cellular protrusions such as the formation of lamellipodia. The link between the mechanical force and the cell signaling pathway could be the stress sensitivity of the

integrin receptors containing β1 chains, which are known to coupled to invasin. Actin would in this case act as a mechanotransducer. Evidence for such a role for integrin has been provided by various groups (Choquet et al., 1997; Ingber, 1991; Nebe et al., 1995; Pommerenke et al., 1996; Wang et al., 1993). In particular, Schmidt et al. (Schmidt et al., 1998) induced local deformation of osteoblastic cells through magnetic beads functionalized with antibodies that bind specifically to integrins exhibiting either β1 or β2 units (or both). They showed that mechanical excitation induces tyrosine phosphorylation of cytoskeletal anchoring proteins and most probably activates MAP kinases and thus couples these proteins to genetic expression pathways.

One likely pathway mediating the growth of an actin cortex is one involving an increase in the Ca^{2+} level, which is known to be accompanied by an increase in the concentration of F-actin (Zhao and Davis, 1999). Another pathway may be mediated by the small GTP-binding protein of the Rho family (Cho and Klemke, 2002), which is known to stimulate pseudopodium formation in the absence of external forces. Our previous results also suggest activation of the Rho GTPases Rho, Rac and CDC42Hs upon binding of invasin-coated beads to β1 integrins (Wiedemann et al., 2001), although no pseudopodium formation, but invagination of the cell envelope followed by the engulfment of the bead, occurs in this case. By contrast, the activation of the Rho GTPase may well promote the growth of the protrusion directed towards the outside of the cell after stimulation by an external force. Consistent with this idea, it was shown recently that application of shear stress to endothelial cells caused Rho activation via αVβ3 integrins (Tzima et al., 2001).

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