Overexpression of myosin IB in living Entamoeba histolytica enhances cytoplasm viscosity and reduces phagocytosis

Sabrina Marion1, Claire Wilhelm2, Heike Voigt3,*, Jean-Claude Bacri2 and Nancy Guillén1,‡

1Unité de Biologie Cellulaire du Parasitisme, INSERM U389, Institut Pasteur, 28 rue du Dr Roux 75724, Paris CEDEX 15, France
2Laboratoire des Milieux Désordonnés et Hétérogènes, UMR7603, and FR2438 CNRS ‘Matière et Systèmes Complexes’, Université Pierre et Marie Curie, 4 Place Jussieu, 75005 Paris, France
3Unité de Pathogénie Microbienne Moléculaire, INSERM U389, Institut Pasteur, 28 rue du Dr Roux 75724, Paris CEDEX 15, France

*Present address: Europäisches Patentamt (EPA), Landsberger Strasse 30, 80339 München, Germany
‡Author for correspondence (e-mail: nguillen@pasteur.fr)

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Summary
The human parasite Entamoeba histolytica is an ancient protozoan that expresses only one unconventional myosin, which has homology with myosin IB from other amoebae. Myosin IB is involved in phagocytosis of human cells by E. histolytica. In this work, we developed a microrheological technique, analysing magnetic phagosomes, which allowed us to probe the density of the F-actin network in living cells. Using this technique, we showed that overexpression of myosin IB led to an increase in cytoplasm viscosity, which correlated with a delay in initiating human cell phagocytosis. To investigate which myosin IB domains sustain cell viscosity changes, we overexpressed truncated forms of the protein. Our results demonstrate that both actin-binding sites that are present in the heavy chain but not the SH3 domain are required to modulate the density of the actin network. These data suggested that, as well as the motor activity, myosin IB in E. histolytica plays a structural role on the actin network owing to its ability to cross-link filaments. The gelation state of cell cytoplasm and the dynamics of cortical F-actin during phagocytosis seem to be modulated by the myosin IB structuring cytoskeleton activity.

Key words: Myosin IB, Viscosity, F-actin, Entamoeba histolytica

Introduction
Actin is an abundant protein in eukaryotic cells that forms filaments (F-actin), which account for many cytoskeletal activities. Cytoplasm isolated from cells forms a gel-like network of F-actin that can solate in the presence of specific actin-binding proteins and calcium (Janmey et al., 1988). Actin gels can be reconstructed in vitro by incubation of actin filaments together with cross-linker proteins. The three-dimensional structure and the physical state of cross-linked actin gels depend critically on the concentration and affinity of the cross-linker (Janmey et al., 1988; Janmey et al., 1990; Wachsstock et al., 1993; Wachsstock et al., 1994; Tseng et al., 2002). A gel cross-linked by purified filamin, a high affinity F-actin binder, behaves like a stiff solid gel with a reduced contractile capacity when the cross-linker is present in high concentration (Janmey et al., 1990; Tseng et al., 2004). By contrast, in the presence of α-actinin or conventional myosin II, an actin gel behaves as a viscoelastic fluid (Wachsstock et al., 1993; Wachsstock et al., 1994; Xu et al., 1998), because these proteins are dynamic F-actin cross-linkers with a high dissociation rate (Humphrey et al., 2002). The actin-gel contraction can be induced in vitro by activated myosin II and occurs simultaneously with the gel-sol transition of the gel (Janson et al., 1991; Kolega et al., 1991). Solation of the actin network by the contractile activity of myosin II has been proposed to play a major role in generating cytoplasmic streaming in Acanthamoeba (Janson et al., 1991; Kolega et al., 1991; Stossel, 1993). In addition, the cytoplasm and the cell cortex have been previously described in vivo as viscoelastic gels (Stossel, 1993; Bausch et al., 1999; Moller et al., 2000; Feneberg et al., 2001) but the role of myosin molecules in the gel mechanical properties still remains unclear. Notably, nothing has been described concerning a role for unconventional myosins.

Class I myosins in Acanthamoeba and Dictyostelium are involved in the formation of pseudopods during cell migration (Titus et al., 1993; Wessels et al., 1996). Furthermore, in many eukaryotic cells, class I myosins have been proposed to mediate, through their MgATPase contractile activity, actin-dependent membrane-based motility such as vesicle trafficking, phagocytosis, macropinocytosis and endocytosis (Novak and Titus, 1997; Mermall et al., 1998; Voigt et al., 1999; Durrbach et al., 2000; Schwarz et al., 2000). Class I myosins are single-headed and consist of a heavy chain and one or more regulatory light chains. The heavy chain presents a highly conserved N-terminal head (the catalytic motor domain) followed by a short neck domain (which binds the regulatory light chains) and a nonhelical C-terminal tail domain. The head domain contains an ATP-sensitive actin-binding site. The tail is divided into three distinct domains: a basic domain (TH1), which binds acidic lipids, a Gly/Pro/Ala-rich (GPA) domain (TH2) and a Src-homology domain (SH3).
that interacts with other proteins. The SH3 domain of myosins IB and IC from Dictyostelium can promote de novo polymerization of actin filaments via the interaction with CARMIL, a protein that binds and activates the Arp2/3 complex (Jung et al., 2001). In Saccharomyces cerevisiae, the heavy chain of Myo3p and Myo5p contains a C-terminal acidic domain that binds directly the Arp2/3 complex (Evangelista et al., 2000; Geli et al., 2000). The TH2 domain binds F-actin in an ATP-insensitive manner (Jung and Hammer, 1994; Liu et al., 2000). The presence of the two actin-binding sites within a single heavy chain has been shown in vitro to be responsible for an F-actin cross-linking property (Fujisaki et al., 1985; Albanesi et al., 1986a). Indeed, the MgATPase activity of myosins IA and IB of Acanthamoeba shows, at high ratios of myosin to actin, an unusual triphasic dependence on F-actin concentration, presumably caused by cooperative cross-linking of actin filaments by myosin I (Albanesi et al., 1985a; Albanesi et al., 1985b; Albanesi et al., 1986b; Ostap and Pollard, 1996). Genetic data from Aspergillus nidulans support these findings. Mutants of the single class I myosin (myoA), which exhibit drastic reduction of the actin-activated MgATPase activity, present normal cell growth and morphology on solid media. Based on these data, it was suggested that myoA in A. nidulans essentially displays a structural role in the cell, rather than a motor function (McGoldrick et al., 1995). In addition, overexpression of myosins IB, IC and IK in Dictyostelium leads to an increase in the cell cortical tension and defects in membrane ruffling (Dai et al., 1999; Schwarz et al., 2000), suggesting that class I myosins regulate the stiffness of the actin-based gel at the cell cortex.

The molecular mechanism by which class I myosins achieve a structural function on the actin network has never been characterized in living cells. Entamoeba histolytica, a primitive eukaryotic cell that causes human amoebic dysentery, is a powerful model for cellular and molecular studies of myosin I. Indeed, bioinformatic genome analyses unexpectedly showed that myosin IB is the only unconventional myosin present in this parasite (The TIGR Entamoeba histolytica Genome Project, 2003; http://www.tigr.org/db/ebk1/eha1). Myosin IB from E. histolytica exhibits the same organization as other amoebic myosins I. In particular, its tail includes the three characteristic domains TH1, TH2 and SH3. The TH2 domain has been shown to be functional for F-actin binding in vitro (Vargas et al., 1997). In resting parasites, myosin IB localized to the cytoplasm and is enriched at the cell cortex. During dissemination in the intestinal tissue and phagocytosis of human host cells, E. histolytica becomes polarized and myosin IB relocates to the pseudopodia, where the network of actin filaments is found in a gelled state (Voigt et al., 1999). Furthermore, E. histolytica cells that overproduce the full-length myosin IB heavy chain exhibit delayed formation of the phagocytic cup, suggesting a defect in the dynamics of cortical actin filaments during phagocytosis (Voigt et al., 1999). Because myosin IB is also localized to the cytoplasm, eventual changes in the organization of the F-actin network in the MyoIB+ strain by either excessive cross-linking or increase in F-actin content might change the cytoplasm’s physical state.

To investigate the molecular mechanism that sustains myosin IB functions in E. histolytica, we overexpressed truncated forms of the heavy chain lacking either one of the two actin-binding sites or the SH3 domain. We set up a micro-rheology technique using magnetic beads that allowed us to probe the mechanical properties of the F-actin cytoskeleton in living cells. We showed that overexpression of myosin IB leads to an increase of cytoplasm viscosity through changes in the density of the F-actin network. Furthermore, we established that both actin-binding sites (located in the heavy chain of myosin IB), but not the SH3 domain, account for these viscosity changes. These data suggest that myosin IB in resting E. histolytica might cross-link actin filaments through the two actin-binding sites within the heavy chain. In addition, we observed that the increase in cytoplasm viscosity directly correlates with a delay in initiating the phagocytic process. Our findings suggest that myosin IB can regulate the dynamics of the F-actin network at the cell cortex, a crucial parameter for fundamental cellular processes such as phagocytosis.

**Materials and Methods**

**Strains and reagents**

E. histolytica HM1:IMSS was cultivated axenically in TY1-S-33 medium (Diamond et al., 1961) at 37°C. Before each experiment, transfected strains were grown for 48 hours in the presence of 50 μg ml⁻¹ G418 in order to induce expression of the transfected constructs.

Amoebic crude cell extract preparation, F-actin labelling with rhodamine phalloidin in fixed cells and the affinity-purified rabbit anti-myosin IB antibody used in this study were previously characterized (Voigt et al., 1999).

**Plasmid construction**

The myosin-IB-encoding gene was previously subcloned into the pExEhNeo vector (Voigt et al., 1999). The deletion of the SH3-domain-encoding sequence (the last 154 nucleotides) was performed by digestion of the pExEhNeo/MyoIB plasmid with the endonucleases BamHI and EcoRV, consequent refilling of ends and DNA ligation. The Myo-tail construct was created by subcloning into the pExEhNeo vector, a fragment generated by the polymerase chain reaction (PCR) carrying nucleotides 2640-3219 of the myoIB tail and elongated at the 5’ end with the VSV tag sequence. The MyoIB deleted for the TH2 domain construct was created by intrahybridization and ligation of two PCR-generated inserts of the nucleotides 1-2850 and 2991-3219, located upstream and downstream, respectively, of the TH2 domain encoding sequence. All constructs were verified by DNA sequencing. Transfection of the plasmids into E. histolytica HM1:IMSS was performed as previously published (Voigt et al., 1999).

**Viscosity measurement**

The magnetic phagosomes were obtained by internalization of 2.8 μm magnetic beads (Dynabeads M-280 tosyl activated; Dynal) coated with human serum for 3 hours. According to published data (Voigt et al., 1999) and immunofluorescence studies, these phagosomes are considered to be late phagosomes that no longer associate with actin microfilaments. Amoebae are then allowed to adhere (for 15 minutes at 37°C) in a glass dish positioned in the magnetic device adapted to the microscope. Phagosomes containing magnetic beads behave as small magnets when placed in a unidirectional magnetic field, attracting each other and forming short chains inside the cytoplasm. We can then impose the rotation of the magnetic pairs in response to the rotation of the magnetic field (B=39 mT). Two approaches have been used, which involved either the study of the response of pairs to an instantaneous 45° rotation (transient response) or to a continuous rotation (permanent response). The first approach has already been described (Wilhelm et al., 2003). Briefly, the two pairs of coils are supplied with the same constant current, creating a static magnetic
field at 45° in the x direction. The field can then align with the x direction within less than 1 microsecond thanks to an electronic commutator. The magnetic probe reacts to the imposed 45° rotation step of the magnetic field by both an elastic response (at short times) and a viscous one (at longer times). We can deduce from the rotational curves obtained, an elastic modulus $G$, a viscous component $\eta$ and a relaxation time $\tau=\eta/G$, this last parameter measuring the proportion of liquid versus solid-like behaviour of the cytoplasm. All the images were captured using an ultrafast camera and digitized on a computer. The video system samples up to 500 images per second. In that condition, the number of sequences is sufficient to follow the motion of the pairs.

In this work, we also developed a second approach in which the magnetic field is uniformly rotated at a given frequency $f$, causing the magnetic pairs to rotate at the same frequency, but their axis lags behind the direction of the field by a constant angle $\phi$ (Fig. 1). The magnetic torque experienced by a bead pair is

$$\Gamma_m = -\frac{\mu_0}{8} M^2 V \sin(2\phi)$$

where $M$ and $V$ are, respectively, the volume magnetization and the volume of a bead. In the steady state, $\Gamma_m$ is only balanced by the viscous torque $\Gamma_v=-2V\kappa\eta$ exerted by the surrounding medium, where $\kappa$ is a geometrical factor. We therefore obtained the viscous modulus $\eta$

$$\eta = -\frac{\mu_0 M^2 \sin(2\phi)}{32\pi f}$$

that allows to infer the local viscosity of the medium from the measured phase lag $\phi$. The geometrical factor $\kappa$ was determined by analysing the rotation of pairs inside a fluid of known viscosity, yielding $\kappa=6.7$.

**Fig. 1.** The rotating magnetic field system to measure the cytoplasm viscosity in living cells. (A) Amoebae that have previously phagocytosed magnetic beads for 3 hours adhered to the bottom of a chamber hermetically sealed containing nutrition medium and maintained at 37°C. The two pairs of magnetic coils are then adjusted within the horizontal plan around the measuring chamber. (B,C) The two types of deformation applied inside living amoebae by the rotation of the magnetic field. (B) 45° step rotation of the phagosome pairs. (C) Permanent rotation at a frequency of $f=0.25$ Hz. In both cases, $\theta$ defines the position of the phagosome pair relative to the $x$ axis: for the 45° step rotation, $\theta$ varies between 0° (initial position of the pair) and 45° (position of the magnetic field). When $t>1$ second, the pair is aligned with the applied magnetic field. In the case of the permanent rotation, $\theta$ varies continuously from 0° to 360° and $\phi$ shows the angular delay between the direction of the phagosome pair and the direction of the magnetic field $B$. The value of this angle directly correlates with the viscosity value of the medium surrounding the pair. (D,E) Image sequences showing the phagosome pair movement inside adherent amoeba, in the case of the 45° step rotation (D) or the permanent rotation (E), in which several magnetic pairs are examined in the same microscope field. (F) Representative creep rotational curve of one pair of phagosome obtained with the 45° step rotation of the magnetic field within the control strain (black) or in cells treated with latrunculin A (grey). The response of the phagosome pair can be described in two steps: an immediate elastic jump, $G$, followed by a viscous relaxation, $\eta$. For the curves depicted in this graph, $G=2.1$ Pa and $\eta=0.27$ Pa seconds in the control strain and $G=2.4$ Pa and $\eta=0.09$ Pa seconds after Lat-A treatment. (G) The complete rotation of a phagosome pair within 4 seconds. Considering that, at $t=4$ seconds, the magnetic field is positioned along the $x$ axis, $\phi$ corresponds to the delay for the pair to reach this axis. In this case, $\phi=18°$.

**Experimental setup**

A magnetic device consisting of two pairs of coils and producing a field $B=39$ mT was adapted to an inverted Leica microscope. The coil pairs were supplied with an alternating current 90° out of phase to generate a magnetic field rotating in a horizontal plane at a frequency of $f=0.25$ Hz. The current is simultaneously fed to a diode, which lights up when the field is parallel to a known direction. The movement of the bead pairs is recorded on videotape and the angle $\phi$ is measured when the diode is on.

**Cytoskeleton fractionation**

2×10⁶ parasites were washed in cold PBS and lysed in 150 μl of lysis buffer (60 mM PIPES, 25 mM HEPES, 125 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 0.5 mM ATP, pH 7.2, containing 1% Triton X-100 and protease inhibitors). Depending on the experiments, amoebae were preincubated with 10 μM jasplakinolide (Molecular Probes, Eugene, OR) for 30 min and then lysed. A magnetic device consisting of two pairs of coils and producing a field $B=39$ mT was adapted to an inverted Leica microscope. The coil pairs were supplied with an alternating current 90° out of phase to generate a magnetic field rotating in a horizontal plane at a frequency of $f=0.25$ Hz. The current is simultaneously fed to a diode, which lights up when the field is parallel to a known direction. The movement of the bead pairs is recorded on videotape and the angle $\phi$ is measured when the diode is on.
Haemoglobin assay
To quantify the erythrocytes ingested by the amoebae, a colorimetric determination of internalized haemoglobin was used (Voigt et al., 1999). Briefly, amoebae (2x10^5) were incubated with human erythrocytes at a ratio (1:100) at 37°C in 0.2 ml culture medium for 15 minutes. The amoebae and erythrocytes were spun down (15 seconds, 8000 g) and resuspended in 1 ml cold distilled water in order to burst uningested erythrocytes. The pellet was washed with PBS and resuspended in 1 ml concentrated formic acid. Samples were measured against a formic acid blank with a spectrophotometer at 400 nm.

Results
Characterizing the biophysical properties of amoebic cytoplasm
The application of magnetic force to living macrophages containing magnetic phagosomes, has been used to demonstrate the role of cytoskeleton in the viscoelastic properties of the cell cytoplasm (Möller et al., 2000). Taking advantage of the phagocytic activity of *E. histolytica*, we addressed the question of the role of myosin IB in the mechanical properties of parasite cytoplasm. We first observed that in *E. histolytica* magnetic beads of 2.8 μm covered by human serum proteins (BHS) were engulfed by phagocytosis, a cytoskeleton-dependent activity. Myosin IB and actin are enriched during the early steps of the process when pseudopodia extend to surround the beads but rapidly disassembles when the phagocytic cup is closed and are no longer detected at later stages of the process (Marion et al., 2000). These phagocytosed magnetic beads reflect equivalent changes in cytoskeleton activity compared with phagocytosed human red blood cells. We chose to use a rotating magnetic field to pilot the movement of the phagosomes, which applied a torque of about 200 kT (kT being the thermal energy: k is the Boltzmann constant and T is the temperature, that is 37°C). This energy is an order of magnitude stronger than that involved in tethering phagosomes to the cytoskeleton by molecular motors. This condition allowed us to neglect the contribution of the phagosome diffusive motion and therefore to examine the viscoelastic parameters of the surrounding cytoplasm. When the unidirectional magnetic field was applied to a population of parasites that have engulfed BHS for 3 hours, magnetic phagosomes behave as small magnets and attract each other, forming pairs inside the cell cytoplasm (Fig. 1A).

To characterize the local mechanical properties of the cytoplasm, we first measured the response of several phagosome pairs to a 45° rotation of a magnetic field of 39 mT (Fig. 1B,D). A representative creep rotational response curve obtained for a pair of phagosomes inside a wild-type parasite is presented in Fig. 1F. We observed that, in terms of its mechanical response to the deformation applied, *E. histolytica* cytoplasm responds with an immediate elastic jump followed by a viscous relaxation. Mean values for elastic and viscous moduli (G and η, respectively) are G=2.2±0.4 Pa and η=0.32±0.05 Pa seconds, and were obtained after examination of 62 different phagosome pairs. The elastic modulus G reflects the instantaneous elastic response. We define the relaxation time τ as the pertinent parameter to quantify the solid versus liquid-like behaviour of the cytoplasm; indeed, a purely elastic medium is characterized by τ=∞, whereas a purely fluid medium by τ=0. Therefore, these data show that the local environment probed by the phagosome pairs in wild-type parasites behaves like a viscoelastic fluid with τ=0.15±0.02 seconds.

Cytoplasmic viscosity reflects the actin network density
To explore the extent to which the actin cytoskeleton contributes to the viscoelastic parameters of the cytoplasm in *E. histolytica*, we repeated the measurements (n=25) in the presence of latrunculin A (Lat A), a drug that depolymerizes F-actin filaments (Fig. 1F). We obtained mean values of G=2.5±0.5 Pa and η=0.13±0.03 Pa seconds. If the elastic modulus is not affected after Lat A treatment, the relaxation time τ fell to 0.06±0.02 seconds, indicating that the cytoplasm is much more liquid-like. These data indicate that actin filament organization appears to be an important parameter modulating the cytoplasm viscoelastic behaviour probed by the magnetic phagosome pairs.

To scan a large population of cells, we modified the technique to facilitate the acquisition of the data. In this approach, the phagosome pair was permanently rotated, under the rotation of the magnetic field at a frequency f=0.25 Hz (Fig. 1C,G). The value of the local viscosity was directly deduced from the phase lag (φ) observed at a given time between the direction of the phagosome pair and the direction of the rotating magnetic field. Consequently, we only measured one angle during the rotation of the pairs simultaneously within many cells visualized in the same microscope field (Fig. 1E). This approach combines the advantage of intracellular rheological techniques (which allow measuring local mechanical properties in a unique cell) with methods taking into account a cell population (which lead to a more accurate statistical value). We then expected to probe slight modifications in the actin network density in *E. histolytica* transfectant cells, which might exhibit different levels of transgene expression.

We first measured the cytoplasmic viscosity in wild-type *E. histolytica* exploring more than 200 magnetic pairs localized to different cells. The values obtained showed a sharp distribution giving a mean of τ=0.29±0.04 Pa seconds, n=203 (Fig. 2A), similar to the one obtained with the 45° rotation experiment. To confirm that this method can also probe
changes in actin network density, we affected the dynamics of actin filaments in living parasites by chemical treatments. We used Lat A and jasplakinolide (Jas), a compound that stabilizes F-actin. Disrupting actin filaments using Lat A led to a more fluid cytoplasm with a lower viscosity: \( \eta = 0.15\pm0.04 \) Pa seconds, a value equivalent to the one previously measured. By contrast, stabilizing actin filaments resulted in an increase of viscosity to \( \eta = 0.46\pm0.04 \) Pa seconds (Fig. 2A). To confirm the alteration of the actin cytoskeleton organization in drug-treated amoebae, we labelled actin filaments using fluorescent phalloidin and examined their localization by confocal microscopy. In the presence of Jas, the cells became flattened and showed increased diffuse fluorescence throughout the cell cytoplasm. By contrast, after incubation with Lat A, cells became round and actin aggregates were observed inside the cells (Fig. 2B). In addition, we quantified the F-actin content of drug-treated cells by cytoskeleton fractionation and quantitative western blots. A fourfold decrease in the F-actin content occurred in the Triton-X-100-insoluble cytoskeleton fraction after the addition of Lat A, whereas a fourfold increase was observed after incubation in the presence of Jas (Fig. 2C). Taken together, these results demonstrate that changes in the cytoplasm viscosity in drug-treated parasites correlate with alterations in the distribution and content of actin filaments. Therefore, measuring the viscosity in the vicinity of the phagosome pairs appears to be indicative of the actin cytoskeleton network density in living \( E. \) histolytica.

\[ \text{E. histolytica strain overexpressing myosin IB showed an increase in cytoplasm viscosity} \]

We have previously constructed an \( E. \) histolytica strain (MyoIB+) that overexpresses the myosin IB protein. These cells contain three times more myosin IB than the wild-type strain or those carrying the control vector plasmid. The MyoIB+ strain is not affected in motility but displayed a delay in the early steps of phagocytosis (Voigt et al., 1999). To investigate whether a threefold excess of myosin IB in \( E. \) histolytica triggers changes on the F-actin network density in vivo, the cytoplasm viscosity was determined as described above. Phagocytosis of magnetic beads was performed in the MyoIB+ strain with a long incubation time, which overcomes the early delay in this process. MyoIB+ cells exhibited a significant increase of cytoplasm viscosity (\( \eta = 0.42\pm0.05 \) Pa seconds) compared with values obtained with the wild-type cells (\( \eta = 0.29\pm0.04 \) Pa seconds) (Fig. 3). Interestingly, the viscosity of MyoIB+ strain cytoplasm is similar to that of cells treated with Jas. Taking into account that the cytoplasm viscosity is affected by the density of the F-actin network, these data indicate that an excess of myosin IB might lead to a denser actin network.

Two actin-binding sites of myosin IB are necessary for changes in cytoplasm viscosity

Overproduction of myosin IB could trigger a modification in the F-actin network organization via two mechanisms: (1) a potential de novo F-actin polymerization activity through the SH3 domain, as previously described for myosin IB in \( Dicystostelium \) (Jung et al., 2001); (2) alternatively, the excess of myosin IB might affect already-polymerized actin filaments.
by an increase of cross-linking activity through its two actin-binding sites. To distinguish between these two possibilities, the role of myosin IB specific domains was ascertained by constructing several truncated forms of the protein (Fig. 4A). First, strains overproducing myosin IB truncated in one of the two actin-binding sites were constructed. One of these constructs (ΔTH2) had a deletion of the actin-binding site present in the tail domain (TH2 domain). The second construct lacked the head domain and the entire tail was fused to a VSV tag (Myo-tail+). This last construct contains the TH2 domain but was devoid of the actin-binding site located within the head domain. Finally, a strain was also constructed with the SH3 domain deleted (ΔSH3). Because it is not yet possible to perform gene replacement in *E. histolytica* by homologous genetic recombination, amoeba strains were generated expressing the truncated forms of the protein along with the endogenous myosin IB. A western blot was then performed to verify the production of the truncated isoforms (Fig. 4B). All the different truncated forms were overproduced at equivalent levels to the full-length protein in MyoIB+ strain and about three times more than in the wild-type cells.

Examination, by magnetic bead microrheological technique, of strains expressing the different constructs showed that the ΔSH3 strain exhibited an increase of the cytoplasm viscosity (η = 0.42 ± 0.05 Pa seconds) that was equivalent to that measured in MyoIB+ strain. By contrast, the strain overproducing myosin IB deleted for the head domain (Myo-tail+) or the ATP-insensitive actin-binding site TH2 (ΔTH2) display the same viscosity value as the control cells.
Entamoeba myosin IB enhances viscosity

**A**

**B**

![Figure 5. The two actin-binding sites in myosin IB heavy chain account for the inhibition of phagocytosis without changes in the F-actin content. (A) Overexpression of myosin IB does not change the F-actin/G-actin ratio compared with the treatment of cells with jasplakinolide. Whole-cell lysates from equivalent cell numbers of the indicated amoeba strain were separated into a Triton-X-100-insoluble fraction, containing actin filaments (F-actin), and a Triton-X-100-soluble fraction, containing globular actin (G-actin). The fractions obtained were analysed by immunoblotting with a monoclonal antibody against actin. The effect of Jas in the wild-type strain on the amount of actin in each fraction was also examined. The MyoIB+ and ΔSH3 strains showed a 10% increase (P<0.01) in the proportion of F-actin on total actin in the cell compared with the other strains examined in this study (P>0.5). By contrast, after incubation with Jas, the proportion of F-actin in the treated amoebae was drastically increased from 22% to 82%. (B) MyoIB+ and ΔSH3 strains show a defect in phagocytosis. Amoebae were incubated with human erythrocytes for 10 minutes at 37°C, lysed and analysed for their haem concentration at 400 nm. The graph depicts the mean optical densities±s.d. of three independent experiments. Only cells overproducing myosin IB or myosin IB deleted for the ΔSH3 domain present a threefold decrease in their phagocytic activity compared with the control strain or the strains overproducing myosin IB deleted for one of the two actin-binding sites.

in the MyoIB+ strain (Fig. 3). This result indicates that the activity of the SH3 domain did not account for changes in cytoplasm viscosity. By contrast, the ΔTH2 and Myo-tail+ strains exhibit a wild-type phenotype. Indeed, viscosity values equivalent to the wild-type cytoplasm were found (η=0.3±0.05 Pa seconds and η=0.3±0.06 Pa seconds for ΔTH2 and Myo-tail+ strains, respectively). The absence of a viscosity increase within these two strains is not due to a reduced protein overproduction level but rather to the absence of the actin-binding sites, because these constructs were found at equivalent quantities to the MyoIB+ strain (Fig. 4B). Based on these data, we conclude that the increase in the cytoplasm viscosity of *Entamoeba histolytica* requires the simultaneous overproduction of the two actin-binding sites present in the heavy chain of myosin IB, but is independent of the SH3 domain.

Overexpression of MyoIB does not change the G-actin to F-actin ratio

To examine whether the increase of the cytoplasm viscosity in the MyoIB+ strain is or is not a consequence of an increase in the polymerization of new actin filaments, we measured the F-actin content after cytoskeleton fractionation of the different parasite strains. By quantitative immunoblotting, the amounts of F- and G-actin were measured in both Triton-X-100-insoluble and Triton-X-100-soluble fractions, respectively (Fig. 5A). The strains ΔTH2 and Myo-tail+ display an equivalent content of F actin, similar to the control cells (P>0.5). A slight increase (P<0.01) in the proportion of F-actin content was revealed in the MyoIB+ and ΔSH3 strains (32% versus 22% for the wild-type cells). Interestingly, the MyoIB+ and ΔSH3 strains exhibited very little change in F-actin content compared with the cells treated with Jas (82%), even though the viscosity increase was almost similar in both cases (η=0.42±0.05 Pa seconds and η=0.46±0.04 Pa seconds for MyoIB+ strain and with Jas, respectively) (Fig. 2A). Based on these data, we suggest that an excess of myosin IB increased the density of the F-actin network without inducing polymerization of new actin filaments.

Changes in cytoplasm viscosity correlates with a defect in phagocytosis

The MyoIB+ strain presents a threefold excess of myosin IB in the Triton-X-100-insoluble fraction and the overproduced protein localizes to the actin-dynamic regions of the parasite such as the cortical region. Previously, we have shown that MyoIB+ strain exhibits a threefold reduction in early steps of the phagocytic process (Voigt et al., 1999). We examined whether the increase in cytoplasm viscosity observed for the MyoIB+ and the ΔSH3 strains can be directly correlated to the phagocytosis defect. After 10 minutes of incubation, we quantified the amount of erythrocytes phagocytosed by the strains overproducing the different truncated forms of the myosin IB. The results showed that only the MyoIB+ and ΔSH3 strains presented a threefold decrease in their phagocytic activity whereas the ΔTH2 and the Myo-tail+ strains phagocytosed the same amount of erythrocytes as the wild-type strain (Fig. 5B). Therefore, the change in cytoplasm viscosity that involved the two actin-binding sites of myosin IB heavy chain directly correlated with a defect in the first step of the phagocytic process.

Discussion

In this study, we analysed the effect of myosin IB overproduction on cytoplasm viscosity and phagocytosis in living *Entamoeba histolytica*. After computer analysis of the 12× completion of genome sequencing in *Entamoeba histolytica*, myosin IB appears to be the only unconventional myosin in this parasite. Previously, we have shown that the overproduction of myosin IB led to a delay in the early steps of phagocytosis, and we have hypothesized that changes in F-actin dynamics account for this delay (Voigt et al., 1999). *Entamoeba histolytica* has a highly motile cell that permanently remodels its cytoskeleton. Indeed, neither actin stress fibres nor cytoplasmic microtubules can be visualized by the usual microscopic techniques, suggesting an unusually high dynamic rate of polymerization and depolymerization of these cellular...
components. Consequently, in this parasite, it is not possible to distinguish a fixed central nuclear region, because the cytoplasmic flows carry the nucleus and intracellular vacuoles away in the direction of cell movement. Thus, we took advantage, first, of natural phagocytic capacities of *E. histolytica* and, second, of the simple cytoskeleton organization of these cells to examine viscosity changes within the parasite cytoplasm, following overexpression of myosin IB. To this goal, we developed a microrheological technique in living amoebae. Translational magnetic forces have been already applied to phagosomes containing magnetic beads within living macrophages and *Dictyostelium* to measure the local viscoelastic moduli of both the membrane and the cytoplasm (Bausch et al., 1999; Möller et al., 2000; Feneberg et al., 2001). These techniques demonstrated the effects of microfilaments, microtubules and associated proteins, such as myosin II, on the mechanical properties of the cytoplasm. From these works, we learnt that diffusive phagosome motion is influenced by the molecular crowding within the cytoplasm and the cytoskeleton tethering of phagosomes by the molecular motors. By contrast, an active phagosome motion can be induced by applying external magnetic forces. In this last case, the resistance of the surrounding medium to the displacement of the phagosome has been proposed to be a direct measure of the viscoelastic properties of the cytoplasm (Möller et al., 2000). The physical approach we developed, based on the use of an external rotating magnetic field, allowed us to perform extensive measurements that resulted in an accurate statistical value for the local cytoplasm viscosity. To investigate whether this technique can indeed probe actin cytoskeleton density, we treated *E. histolytica* cells with Lat A, which led to a drastic decrease in the actin polymer content. Using the rheological approach, we measured a decrease in cytoplasm viscosity in Lat-A-treated cells. Also, treatment with Jas (which stabilizes actin filaments and increased the amount of F-actin about fourfold) increased the cytoplasm viscosity.

We conclude that the magnetic phagosomes under the magnetic field rotation acted as sensitive probes for measuring changes in the actin cytoskeleton density, reflected by changes in cytoplasm viscosity. Interestingly, a similar increase in cytoplasm viscosity was measured in MyoIB+ strain and cells treated with Jas, whereas the amount of actin filaments in MyoIB+ cells was not significantly different from the wild-type strain. In addition to these data, we found that the increase in cytoplasm viscosity in MyoIB+ strain was not affected by the presence of the SH3 domain of the myosin IB heavy chain, because a strain overproducing the protein deleted for this domain still exhibited a denser cytoplasm. By contrast, cell viscosity enhancement is dependent on the simultaneous presence of the two actin-binding sites within the heavy chain. Together, these data suggest that, in resting cells, myosin IB overproduction might enhance the cross-linking of pre-existent actin polymers rather than inducing de novo actin polymerization. Our data are consistent with results obtained in vitro, which demonstrated that myosins IA and IB from *Acanthamoeba* cross-link actin filaments via the two actin-binding sites within a single heavy chain molecule (Fujisaki et al., 1985; Albanesi et al., 1986a/b). In addition, kinetic studies of the ATPase cycle of myosins I demonstrated that, most of the time, the protein is weakly bound to the actin filaments (Albanesi et al., 1985b; Ostap and Pollard, 1996). Therefore, it has been proposed that previous cross-linking of actin filaments would be necessary to create highly concentrated acto-myosin I bundles capable of supporting the cortical contraction when myosin I molecules are activated (Albanesi et al., 1985b; Ostap and Pollard, 1996). Experimental data from *Acanthamoeba* fit well with this hypothesis because no activation of myosin I molecules by phosphorylation of the heavy chain is necessary to cross-link actin filaments in vitro. Such a preformed gel enhances the MgATPase contractile activity of added activated myosin I (Fujisaki et al., 1985; Albanesi et al., 1986a). Furthermore, based on in vivo observations, the putative structural role of myosins I in cells has been emphasized. Indeed, in *A. nidulans*, point mutations in the consensus phosphorylation site of myoA heavy chain (which reduces the MgATPase activity in vitro to 1% of the wild type) have little effect on growth and development (McGoldrick et al., 1995). These data allowed the conclusion that the essential role of myosin I in this fungus is mostly structural and does not require the motor activity. In addition, myosin IK in *Dictyostelium* presents a distinct architecture compared with other class I myosins. This myosin consists of the head domain with the consensus phosphorylation site in its actin-binding surface and a short neck domain but, strikingly, lacks the characteristic tail domain and therefore does not contain a SH3 domain. A TH2-like domain is present as an insertion loop within the head domain. Overexpression of myosin IK leads to increases in cortical tension and defects in membrane ruffling. Based on these data, it was suggested that myosin IK can act as an F-actin cross-linker at the cell cortex. Interestingly, MyoK+ cells exhibit a similar phenotype to MyoIB+ cells in *E. histolytica*, because motility is not affected but a reduced initial rate of phagocytosis is observed. In *E. histolytica*, myosin IB is also enriched at the cell cortex (Voigt et al., 1999). We observed that MyoIB+ and ΔSH3 strains that present an increase in cytoplasm viscosity also exhibit a delay in initiating the extension of the pseudopodial appendages during phagocytic cup formation. This delay was not observed in cells that produced myosin IB protein deleted for the TH2 domain. These data suggest that overproduction of myosin IB might increase the rigidity of the cortical actin filament network by its potential cross-linking property, causing a decrease in the flexibility of the plasma membrane.

Nevertheless, class I myosins are considered to be molecular motors providing the power for contraction at the cell cortex. In *Dictyostelium*, strains overproducing full-length myosin IB exhibit defects in actin-based membrane motility processes such as chemotaxis and pinocytosis. Phosphorylation of the heavy chain and the presence of the SH3 domain are both necessary to achieve these functions in vivo (Novak and Titus, 1997). In addition, during cortical actin-patch assembly in *S. cerevisiae*, it has been shown that myo5p and myo3p induce de novo actin polymerization via their SH3 domain and that the motor activity of these myosins is required for the actin polymerization step (Letcher et al., 2000). These data suggest that the de novo actin polymerization activity can be activated simultaneously with the motor activity. In contrast to *Dictyostelium* or *S. cerevisiae*, *E. histolytica* contains only one unconventional myosin. Therefore, we hypothesize that this single myosin has different functions in this parasite. Interaction between human erythrocytes and specific *E. histolytica* surface receptors might induce the signalling pathway that triggers the
switch from the potential cross-linking function of myosin IB in resting cells to the contractile activity during phagocytosis. Taking these data together, myosin IB in E. histolytica appears to be an interesting protein that might exhibit dual functions to sustain actin network dynamics. (1) As a cross-linker via both actin-binding sites, myosin IB can participate in the maintenance of the cortical tension and the gelation state of the cytoplasm in such a way that cells can move and react to internal or external forces without requiring active signals. (2) Under signal-induced conditions (e.g. during phagocytosis) myosin IB acts as a molecular motor that sustains the actin-based contraction of the plasma membrane during phagocytic cup formation. The fact that myosin IB is the only unconventional myosin in E. histolytica, opens perspectives to understand how these different functions are regulated in the cell, in particular during phagocytosis of human cells, a major factor in the virulence of amoebiasis.

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