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BFA-sensitive and insensitive exocytic pathways in *Entamoeba histolytica* trophozoites: their relationship to pathogenesis

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Summary

***Entamoeba histolytica* manifests its pathogenicity through several cellular processes triggered by external stimuli that activate signal transduction pathways. The intense secretory activity resulting from stimulation is not correlated with a typical endoplasmic reticulum (ER) or Golgi organization, and little is known in this parasite about endocytic/exocytic pathways. The interactions of trophozoites with fibronectin (FN) and cultured mammalian cells, which elicit secretory activities, were chosen to study mechanisms that regulate cytoplamic traffic. Results showed that Brefeldin A (BFA) induced redistribution of the vesicular network recognized by antibodies against amoebic proteins PDI and ERD2. Furthermore, BFA diminished traffic to the plasma membrane of the β1 integrin-like FN receptor and the heavy subunit of the Gal/GalNAc lectin, required for adhesion to FN and target cells, respectively. However, BFA did not prevent thiol-proteinase secretion or inhibit the traffic of *de novo* synthesized proteinases. These data suggest that two distinct transport systems occur in *E. histolytica*, one similar to classical membrane protein transport and another independent of BFA and inducible by external stimuli. Actin-myosin contractility of the cortical cytoskeleton seems necessary for the final release of exported proteinases and the proper function of the surface proteins involved in adhesion.**

Introduction

Trophozoites of *Entamoeba histolytica* invade the intestinal mucosa of their human host and produce lesions in the intestine and other organs. The mechanisms involved in the entrance of amoebae to the mucosa as well as those responsible for damage to tissues are not yet fully understood (Perez-Tamayo *et al*., 1990; Espinosa-Cantellano and Martinez-Palomo, 2000).

Amoebic pathogenicity is manifested throughout several processes that depend on trophozoite motility. In addition to specific molecules directly involved in virulence, locomotion, adhesion, endocytosis, secretion, and membrane plasticity are necessary for engulfment and killing of bacteria, internalization of erythrocytes, lysis of target cells, and degradation of extracellular matrix (Ravdin and Guerrant, 1981; Mirelman, 1987; Talamas-Rohana and Meza, 1988; Leippe, 1997; Vines *et al*., 1998; Que and Reed, 2000). All these processes are triggered in the presence of external stimuli that activate signal transduction pathways coupled to membrane receptors and other surface proteins. As a result of this activation, amoebae reorganize their cytoarchitecture, metabolic routes, and gene expression (Manning-Cela and Meza, 1997; Meza, 2000; Ortiz *et al*., 2000; Katz *et al*., 2002).

Secretion is one of the processes, thought to be particularly important in virulence, that is modified by external stimuli and signal transduction (Meza, 2000; Que and Reed, 2000; Moncada *et al*., 2003). Amoebic proteins involved in adhesion like the Gal/GalNAc lectin, as well as other surface or secreted proteins such as the amoebapore, the cysteine-rich Ariel, and cysteine proteinases contain signal sequences to be transported to the plasma membrane (Mann *et al*., 1991; Bruchhaus *et al*., 1996; Leippe, 1997; Mai and Samuelson, 1998). Recently, genes have been cloned for ERD2, a *cis*-Golgi-associated protein, and for BiP, protein disulfide isomerase (PDI) and calreticulin, which are involved in protein translocation in the lumen of the ER (Sanchez-Lopez *et al*., 1998; Field *et al*., 2000; Ramos and Alagon, 2000; Gonzalez *et al*., 2002). Furthermore, endosomal vesicles containing acid phosphatase, cysteine proteinases, and Rab-like proteins have been isolated (Temesvari *et al*., 1999). However, knowledge about the mechanisms involved in cytoplasmic traffic and secretion is just beginning to be unraveled. Electron microscopy studies have shown that ribosomes form clusters that could correspond to rough ER, although membranous material does not seem to hold them together. Individual lamellae or lamellar stacks corre-

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sponding to a Golgi system have not been observed, even using rapid freezing and cytochemical methods. Instead, vacuolar and occasionally tubular structures without defined distribution are the most conspicuous structures in the cytoplasm (Mazzuco *et al*., 1997; Ghosh *et al*., 1999; Chavez *et al*., 2000).

It has been previously reported by us and by J. Samuelson's group, who also identified gene sequences for ARF in this parasite, that trophozoites are sensitive to Brefeldin A (BFA), a drug that disrupts the Golgi system in many eukaryotic cells (Ghosh *et al*., 1999; Manning-Cela *et al*., 2000). These observations together with the data described above suggest the presence of endocytic and exocytic pathways in *Entamoeba histolytica* with features common to those operating in other eukaryotic cells, but with distinctive features that need to be better analysed.

We have used the *in vitro* models of fibronectin (FN) substrate degradation and damage to cultured mammalian cells to investigate cytoplasmic traffic and secretion pathways in *E. histolytica*. These effects reflect the active induction of proteinase secretion, which is known to occur after adhesion of trophozoites to FN substrates or to cell surfaces (Talamas-Rohana and Meza, 1988; Franco *et al*., 1999). Our results show that the amoebic proteins EDR2 and PDI are found in the cytoplasmic vesicular network present in trophozoites and are redistributed by BFA treatment. BFA had a deleterious effect on adhesion and damage to target cells but did not affect thiol-proteinase release, induced by the presence of FN. In contrast, traffic of proteins destined for the plasma membrane like the heavy subunit of the Gal/GalNAc lectin and the β1 integrin-like FN receptor was sensitive to BFA, suggesting that vesicles containing these proteins move through a classical membrane secretory pathway. Our data also indicate that the final release of exported proteinases is modulated by the cortical cytoskeleton, since inhibition of actin–myosin interaction with 2,3-butanedione monoxime (BDM) rendered amoebae incapable of thiol proteinase secretion. The specific action of BDM is supported by its inhibition of phagocytosis, pinocytosis, and cap formation, all of them processes that require myosin II participation. Furthermore, BDM blocked the release of internalized dextran-loaded vesicles. The impaired ability of amoebae to adhere to cells or to FN substrates in the presence of BDM, even though the appropriate proteins were present on the parasite's surface, could partially result from the alteration of the cortical cytoskeleton function.

Results

Brefeldin A has a disruptive effect on the putative ER and Golgi vesicular network

Recent electron microscopy studies have shown that vesicles and tubular structures in the cytoplasm of *E. histolytica* and *E. invadens* trophozoites contain components that have been identified in the ER and Golgi of several eukaryotic cells (Ghosh *et al*., 1999; Temesvari *et al*., 1999). *E. histolytica* genes encoding two proteins associated with these structures, ERD2 and PDI, were cloned recently by the group of A. Alagon (Sanchez-Lopez *et al*., 1998; Ramos and Alagon, 2000). Specific monoclonal antibodies to these proteins, prepared by the same group, were utilized to assess the effect of BFA on the distribution of putative ER and Golgi vesicles. Figure 1 shows trophozoites containing many vesicles that bind antibodies to ERD2 (A and B) and PDI (Figs 1C,D). These vesicles were heterogeneous in size and randomly distributed in the cytoplasm. In occasional cells, positive vesicles localized in the vicinity of the nucleus. Treatment of trophozoites with BFA caused visible changes in the distribution of labeled vesicles. As seen in Figs 1(B, D), the vesicles in BFA-treated cells merged into clusters and elongated aggregates. This effect, however, did not resemble the dispersion of the Golgi described after BFA treatment in other eukaryotic cells (Burdett, 2002; Tamaki and Yamashina, 2002).

Brefeldin A inhibits adhesion of trophozoites to FN substrates but has no effect on their degradation

To gain insight into the mechanisms that could operate in trophozoites during cytoplasmic trafficking and exocytosis, we examined the *in vitro* interaction of trophozoites with FN substrates. The specific interaction of the para-

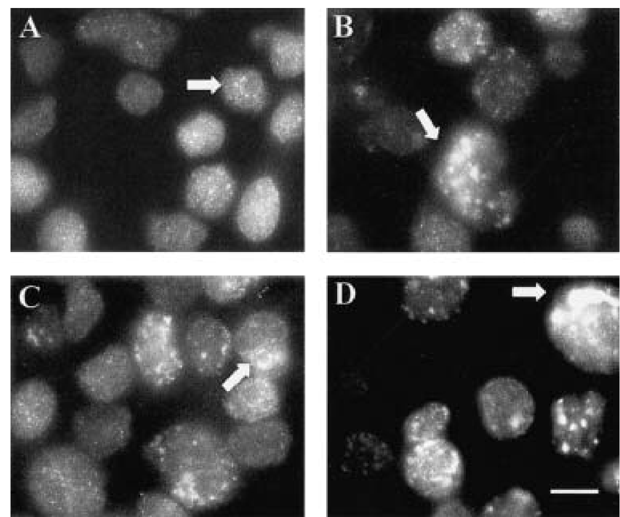

Fig. 1. Immunolocalization of vesicles associated to ERD2 and to PDI. Trophozoites in regular culture medium (A,C). Trophozoites in culture medium containing 75 μg ml^{-1} BFA (B,D). Trophozoites labeled with antibodies to ERD2 (A,B) and to PDI (C,D). Arrows indicate cells in which labeled vesicles were localized around the nucleus (A,C) or forming clusters and elongated aggregates (B,D). Bar = 20 μm.

site with this extracellular matrix (ECM) protein stimulates signal transduction and focal release of proteinases together with locomotion and chemotaxis, all of which require active participation of the cytoskeleton (Talamas-Rohana and Meza, 1988; Santiago *et al.*, 1994; Franco *et al.*, 1997; Manning-Cela and Meza, 1997; Soid-Raggi *et al.*, 1998). As a first step, the effect of BFA on the binding of trophozoites to FN-covered surfaces was evaluated. Figure 2 (A) shows that adhesion of control amoebae to FN is at least 2-fold greater than adhesion to BSA-covered plastic surfaces. BFA treatment of the same cell population produced a clear inhibitory effect of the FN stimulus, as adhesion capabilities of drug-treated trophozoites decreased almost 45%. The weaker non-specific adhesion to BSA-covered plates, was also reduced by BFA.

Figure 2(B) shows the kinetics of release of ^{125}I-labeled FN fragments by trophozoites cultured in normal medium or in the presence of BFA. Degradation of the FN substrate was observed within 15 min after adhesion; the release of labeled fragments at this time represented about 20% of the total radioactivity released after 2.5 h. Surprisingly, the kinetics of release of ^{125}I-labeled FN fragments produced by trophozoites incubated in the presence of BFA was similar to that caused by amoebae cultured in the absence of the drug.

These data indicate that while adhesion of trophozoites to FN is inhibited by BFA, the degradation of FN substrates is not, suggesting that the release of active proteinases is independent of a BFA-regulated exocytic pathway.

Spontaneous release of proteinases could explain their secretion in the presence of BFA, which has an inhibitory action upstream in the exocytic pathway. We examined this possibility by metabolically labeling amoebae with ^{35}S-methionine for 2 h in absence or presence of BFA, followed by a chase of 2.5 h to determine the destiny of newly synthesized proteins. As shown in Fig. 2(C), we found no difference in the release of radiolabeled proteins between BFA-treated and control amoebae. Thus, the presence of the drug did not affect the cytoplasmic traffic of *de novo* synthesized proteins released to the medium. No significant differences were found in the incorporation of ^{35}S-methionine into the TCA-precipitable material obtained from cell homogenates of control or BFA-treated trophozoites (data not shown). The zymogram in Fig. 2(D) shows the released proteinase activities, which corresponded mainly to thiol proteinases. The release of these activities was not modified by increasing concentration of BFA. These results show that BFA did not affect the synthesis of thiol proteinases and support the possibility that their release follows an independent pathway that can be induced by external stimuli.

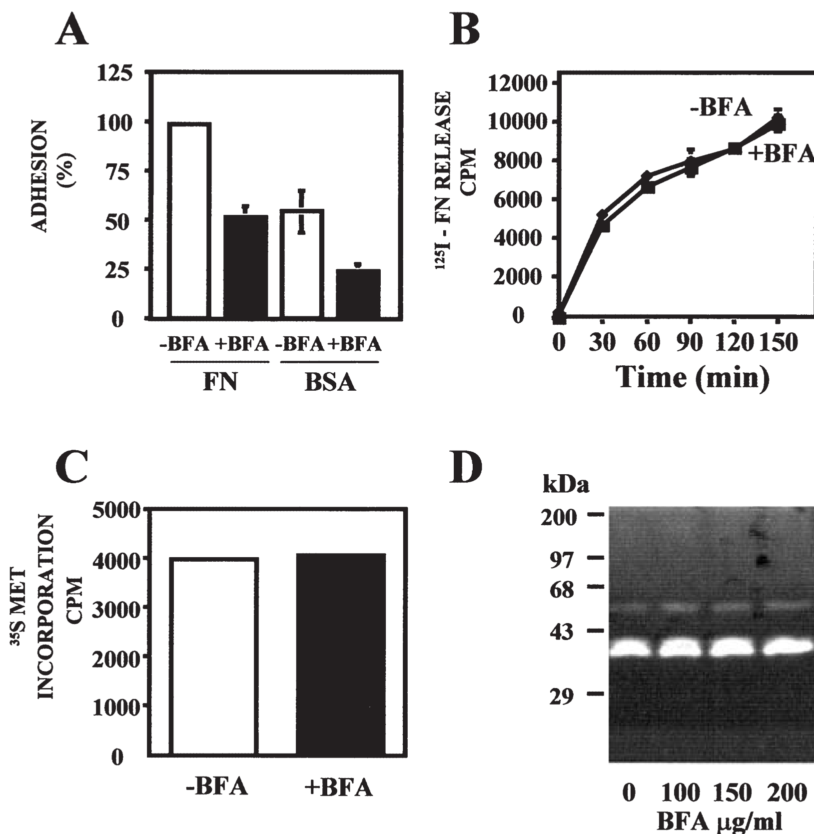

Fig. 2. Adhesion of untreated (–BFA) and treated (+BFA) trophozoites to FN substrates and release of ^{125}I –FN fragments. (A) Adhesion of untreated and BFA-treated cells was quantified after 20 min of interaction as indicated in Materials and Methods. Values are means ± SD of sixtuplicates from three independent experiments. (B) Kinetics of degradation of ^{125}I-labeled FN. Each point represents the radioactivity released by –BFA and +BFA trophozoites from three independent experiments. (C) ^{35}S-methionine incorporation into TCA-precipitable material by –BFA and +BFA trophozoites. Results represent values from two independent experiments. (D) Zymogram of a gelatin-polyacryalamide gel developed to reveal thiol proteinase activities in the culture medium from control (O) and BFA-treated trophozoites. The gel is representative of results obtained in 5 experiments.

Brefeldin A inhibits cell damage by trophozoites

The cysteine proteinases released by trophozoites are considered to be important participants in amoebic virulence and destruction of target cells (Ankri *et al*., 1999; Que and Reed, 2000; Zhang *et al*., 2000; Moncada *et al*., 2003). Since they are released to the medium independently of the perturbation of cytoplasmic traffic caused by BFA, it was surprising that the damage inflicted on cultured MDCK cells was reduced 46% in the presence of the drug, as shown in Fig. 3(A). This observation suggested that the reduction in the cytopathic capabilities of trophozoites produced by BFA must result from inhibition of some other cell process necessary for cell damage.

As mentioned above, membrane–cytoskeleton interactions responsible for trophozoite motility are also stimulated by the interaction of trophozoites with FN substrates and target cells. It has already been shown that treatment of amoebae with cytochalasin D disrupts their actin cytoskeleton and renders rounded amoebae incapable of adhering to FN or damaging target cells (Ravdin and Guerrant, 1981; De la Garza *et al*., 1989). We therefore, assessed the effect of BFA on pinocytosis and phagocytosis, functions implicated in pathogenicity and known to be regulated by the cortical actin cytoskeleton. As shown in Fig. 3(B), neither process was significantly modified in trophozoites treated with the drug, indicating that BFA-mediated inhibition of cell damage was not caused by alteration of cortical cytoskeleton function.

FN receptor and Gal/GalNAc Lectin export to the plasma membrane is sensible to BFA

We next examined the possibility that BFA was affecting

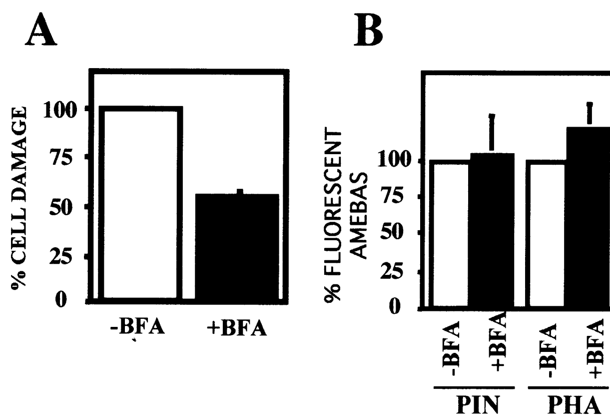

Fig. 3. Effect of BFA on cytotoxic, pinocytic and phagocytic capabilities of trophozoites. (A) Cell damage to confluent MDCK monolayers caused by trophozoites in absence (–BFA) or presence (+BFA) of BFA. (B) Pinocytosis of FITC-dextran and phagocytosis of GFP-expressing bacteria in untreated (–BFA) and BFA-treated trophozoites (+BFA). Values are means ± SD of triplicates from three independent experiments.

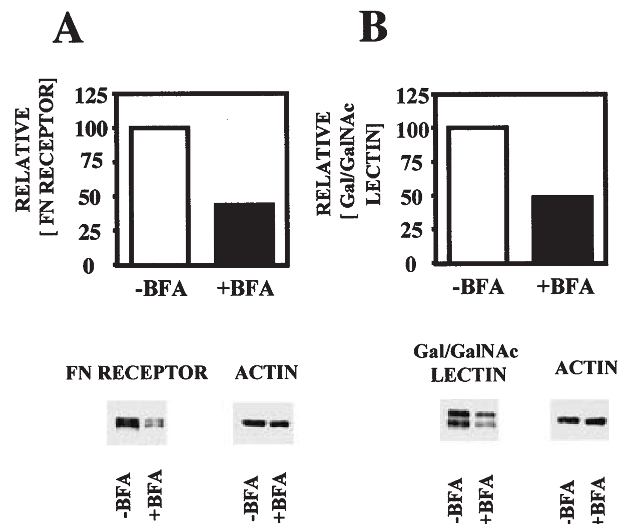

Fig. 4. Quantitative immunoblotting of the FN receptor and of the heavy subunit of the Gal/GalNAc lectin after PAGE of plasma membrane fractions. Proteins from untreated (–BFA) or BFA-treated amoebae (+BFA) were reacted with specific monoclonal antibodies to the FN receptor or to the lectin (A and B, respectively). Quantification was done by densitometry and is expressed as relative values to those of the same proteins in untreated amoebae. A representative immunoblot and the corresponding quantification are shown. Actin was utilized as internal control for the protein concentration in each lane.

the transport of proteins that play an important role in the initial steps of pathogenic behavior, such as adhesion to target cells and substrates. Two proteins known to be involved in these functions are the Gal/GalNAc lectin and the $\beta 1$ integrin-like FN receptor. The presence of these proteins in plasma membrane fractions of control and BFA-treated amoebae was analyzed by immunoblot using antibodies specific for the FN receptor and the heavy subunit of the Gal/GalNAc lectin (known to contain the domain for binding galactose residues). As shown in Fig. 4, quantitative immunoblotting of plasma membrane fractions from control and BFA-treated trophozoites revealed that treatment with BFA reduced the amount of the two proteins present in plasma membranes to about half of normal levels. This finding indicates that these two plasma membrane proteins, which earlier data have shown play a role in tissue damage by trophozoites (Vines *et al*., 1998; Dodson *et al*., 1999; Sengupta *et al*., 2001), are transported through a BFA-sensitive exocytic pathway.

Cytoskeleton protein disruption affects adhesion, cell damage, and protein release

Cortical cytoskeleton dynamic function is required for protein secretion. Most studies have made use of actin microfilament disruption as a tool to block exocytosis at several levels (Verrey *et al*., 1995; Trifaro *et al*., 2000).

Since trophozoites treated with cytochalasins round up and detach from the substrate, we utilized 2,3-butanedione monoxime (BDM) to investigate cortical cytoskeleton participation in the protein traffic process. BDM specifically inhibits actin-myosin-driven contractility by blocking the ATPase activity of myosin II, but does not cause depolymerization of actin filaments or visible structural changes in the organization of the cortical cytoskeleton (Cramer and Mitchison, 1995; Castillo *et al.*, 1998 and 2002). We found that treatment of trophozoites with BDM for 60 min did not cause visible disorganization of actin. In both control (Fig. 5A) and BDM-treated cells (Fig. 5B), actin appeared diffuse in the cytoplasm and concentrated in the cortical region, in particular in what seemed to be pinocytic invaginations. However, BDM caused a 57% decrease in adhesion of amoebae to FN substrates and an almost complete inhibition of cell damage (Figs 5C,D). Furthermore, BDM rapidly inhibited the release of ^{125}I-labeled FN fragments after trophozoite contact with this substrate. During a 2 h period, BDM-treated amoebae produced less than half the amount of FN degradation as control trophozoites (Fig. 5E). Furthermore, BDM practically shut down the release of thiol proteinases; barely detectable activities for these enzymes were revealed in gelatin zymograms of culture media from BDM-treated amoebae after 2 h (Fig. 5F). These results indicated that actin–myosin interaction is required for adhesion of trophozoites to cells and FN-substrates and that proper function of the actin-myosin cytoskeleton is also a requirement for the release of thiol proteinases.

Inhibitory Effect of BDM in Myosin-dependent functions

To test BDM specificity for myosins of *Entamoeba histolytica*, we analyzed cellular processes that in this parasite have been clearly demonstrated that require the participation of myosin I and myosin II (Arhets *et al.*, 1995 and 1998; Voigt *et al.*, 1999). As shown in Fig. 6, cap formation,

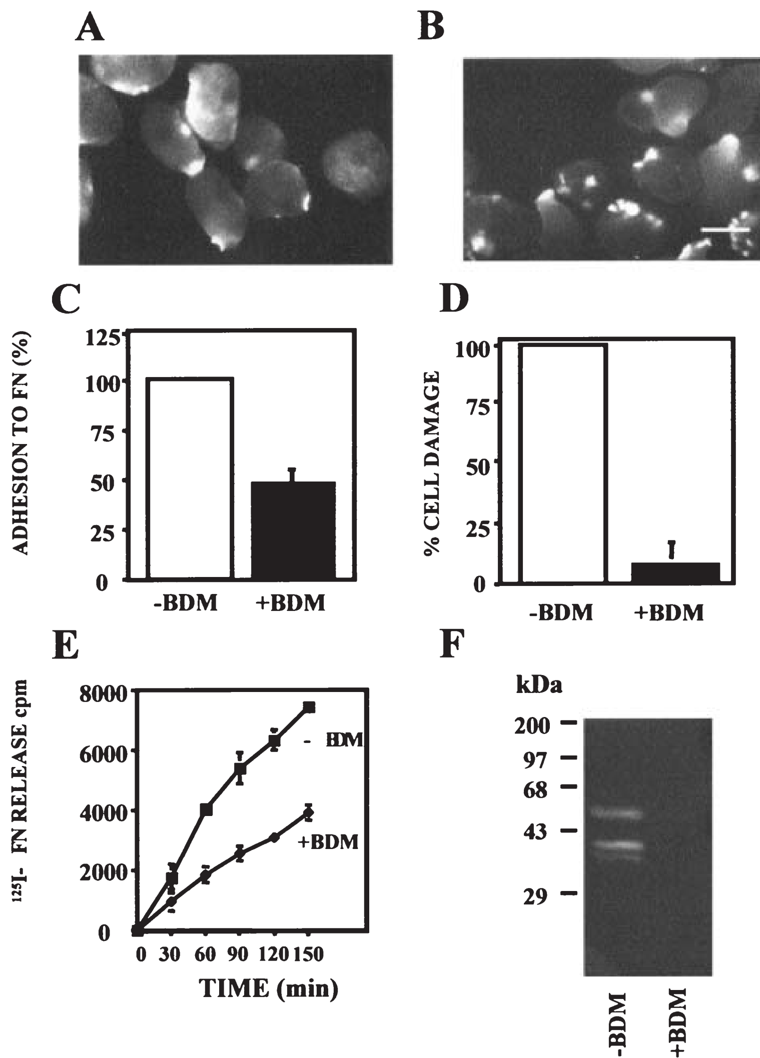

Fig. 5. Disruption of actin–myosin interaction and its effect on adhesion to FN, cell damage and protein release. Actin was stained with Rhodamine-phalloidin in untreated (A) and BDM-treated trophozoites (B). Actin appears diffuse in the cytoplasm and concentrates at sites of pinocytosis. (C) Adhesion to a FN substrate by untreated (–BDM) and BDM-treated trophozoites (+BDM). Values are means ± SD of sixtuplicates from three independent experiments. (D) MDCK cell damage by untreated (–BDM) or BDM-treated trophozoites (+BDM). Values are means ± SD of triplicates from three independent experiments. (E) Kinetics of degradation of ^{125}I-FN by untreated and BDM-treated trophozoites. Each point represents three separate experiments done in triplicate. (F) Zymogram of thiol proteinases released to the culture medium by untreated (–BDM) and BDM-treated trophozoites (+BDM). The zymogram is representative of results obtained in three independent experiments. Bar = 20 μm.

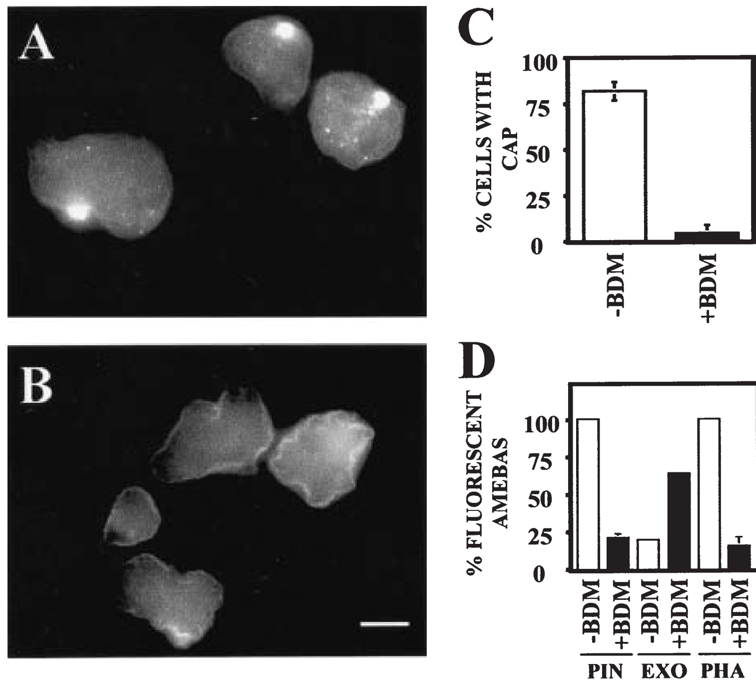

Fig. 6. Effect of BDM on capping, pinocytic, exocytic and phagocytic capabilities of trophozoites. Capping was induced by ConA-FITC in untreated (A) and BDM-treated trophozoites (B). The proportion of cells with Cap among the total untreated (–BDM) and BDM-treated trophozoites (+BDM) was determined from 10 random fields in two independent experiments (C). (D) Pinocytosis of FITC-dextran (PIN), exocytosis of Alexa 488-dextran (EXO) and phagocytosis of GFP-expressing bacteria (PHA) in untreated (–BDM) and BDM-treated trophozoites (+BDM). Values are means ± SD of triplicates from three independent experiments.

as well as pinocytosis, exocytosis and phagocytosis, were significantly inhibited by BDM. Figure 6 shows that caps formed in 85% of the trophozoites after Concanavalin A induction (Figs 6A,C), were almost absent if the same population was treated with BDM (Figs 6B,C). Figure 6(D) shows that pinocytosis, exocytosis and phagocytosis are also impaired in the presence of BDM. Pinocytosis and phagocytosis have also been shown to be blocked by cytochalasin D, a drug that disrupt the actin organization in the parasite (Ravdin and Guerrant, 1981; De la Garza *et al.*, 1989). The present results corroborate previous observations indicating the participation of cortical actin and myosin II in cap formation (Espinosa-Cantellano and Martinez-Palomo, 1994; Arhets *et al.*, 1995 and 1998) and support their participation in other processes requiring a dynamic interaction between the plasma membrane and the cortical cytoskeleton.

Disruption of the cortical cytoskeleton does not affect FN receptor and Gal/GalNAc lectin export to the plasma membrane

To evaluate if the marked decrement in adhesion and cell damage caused by BDM treatment could result from defective traffic of the FN receptor or the Gal/GalNAc lectin to the plasma membrane or as a consequence of impaired cortical cytoskeleton function, we monitored levels of the lectin and the FN receptor in plasma membranes of BDM-treated and control trophozoites. Immunoblots and quantitative measurements of the two proteins carried out using the monoclonal antibodies against the heavy subunit of the Gal/GalNAc lectin and the β1 integrin-like FN receptor are shown in Fig. 7. Densitometric analyses from three independent experiments revealed that in contrast to the results obtained with BFA-treated amoebae shown in Fig. 4, the plasma membranes of BDM-treated trophozoites contained a higher concentration of the two proteins. Figure 7 shows the results obtained with one of the immunoblots, in which the concentration of the FN receptor and the lectin were elevated by 23 and 32%, respectively, in BDM-treated amoebae relative to control cells. These results suggest that these two proteins are being accumulated in the plasma membrane of drug-treated cells.

In spite of the accumulation of the Gal/GalNAc lectin and the FN receptor in the plasma membrane, amoebae treated with BDM are strongly impaired in adhesion to cells and FN-substrates, and also in proteinase release as shown in Fig. 5. Thus, actin–myosin interaction seems necessary for adequate function and recycling of surface molecules involved in adhesion to cells and FN substrates. We infer myosin II in the cortical cytoskeleton inhibits actomyosin-based dynamics of membrane components, blocking the retrieval of surface proteins as well as the release of exported molecules.

Discussion

In *Entamoeba histolytica* trophozoites, cytochemical reactions and antibodies have been used to identify proteins known to be associated with ER and Golgi-related structures such as phosphatases, ARF, and εCOP. These

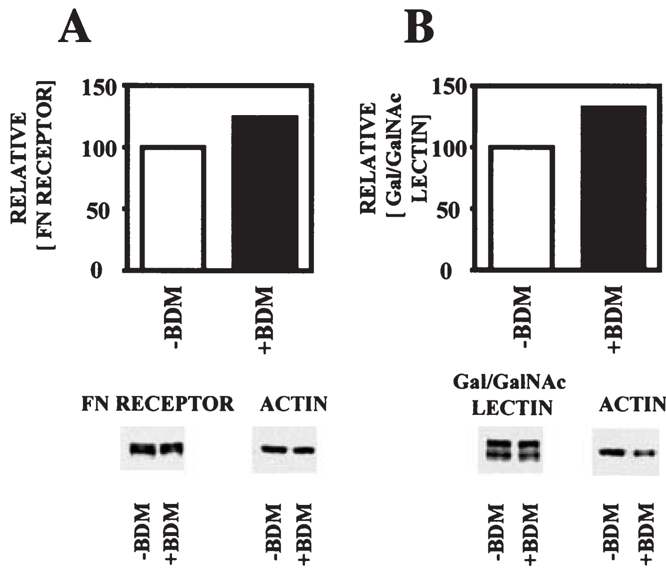

Fig. 7. Quantitative immunoblotting of the FN receptor and of the heavy subunit of the Gal/GalNAc lectin in the absence (–BDM) or presence of BDM (+BDM). Plasma membrane proteins were reacted with the corresponding specific monoclonal antibodies. Quantification was done by densitometry and is expressed as relative values to those of the same proteins in untreated amoebae. Actin was used as internal standard for the protein concentration in each lane.

studies have indicated that these systems mainly consist of vesicles of different sizes dispersed in the cytoplasm (Mazzuco *et al.*, 1997; Ghosh *et al.*, 1999; Temesvari *et al.*, 1999). This unusual structural organization may require distinctive trafficking mechanisms which need to be determined.

In the present work, we have used antibodies against the amoebic proteins ERD2, an ER retention receptor that is localized in the *cis*-Golgi, and PDI, which is retained in the ER lumen. These antibodies labeled numerous vesicles of different size that were not localized to a restricted part of the cytoplasm. Contrary to what has been reported for the small number of vesicles labeled with ARF in *Entamoeba invadens,* EDR2 and PDI-positive vesicles merged into larger vacuoles upon treatment of the trophozoites with BFA, a drug widely used to disrupt cytoplasmic traffic in many eukaryotic cells, although its specific target on the ER-TGN path is still undetermined (Chavrier and Goud, 1999; Shinotsuka *et al.*, 2002). In a previous study we reported that *E. histolytica* trophozoites stopped growing at a BFA concentration of 75 μg and died within 24 h at higher concentrations (Manning-Cela *et al.*, 2000). These observations indicated that trophozoites have secretory pathways important for survival that are sensitive to BFA and that this drug can be used to study vesicular traffic in this parasite.

The secretory pathways of two types of proteins were thus explored; (1) thiol proteinases, known to be responsible for the degradation of ECM proteins and therefore possibly involved in the invasion of tissues by the parasite and (2), two proteins destined to the plasma membrane that are key participants in the binding of trophozoites to target cells and substrates, a necessary step in the pathogenic process.

As thiol proteinases are released by trophozoites under several stimuli, one of which is the interaction with FN, the *in vitro* model of binding and degradation of FN substrates by trophozoites was utilized in this study (Talamas-Rohana and Meza, 1988). The trophozoites bind to the FN substrate through special actin adhesion structures that also function as signal transduction organelles, and facilitate focal degradation of FN at the sites of contact (Vazquez *et al.*, 1995; Meza, 2000). The present study demonstrated that the binding of trophozoites to FN was significantly inhibited by BFA, but that this drug had no effect on the release of thiol proteinases and the rate of degradation of the FN substrate, suggesting that proteinases are secreted by a BFA-independent pathway. The finding that the release of *de novo* synthesized proteinases into the culture medium was also not sensitive to BFA supports the hypothesis that vesicles carrying thiol-proteinases travel by an alternate route and are released as response to external stimuli.

However, as adhesion to FN, that requires the presence of an active surface receptor, was significantly reduced in trophozoites treated with BFA, we analyzed the binding of trophozoites to mammalian cells, which is also mediated by an amoebic surface receptor. In this case, a Gal/GalNac lectin binds to galactose residues on the mammalian cell surface. We found that BFA also had a strong inhibitory effect on both the binding of the trophozoites to cells and the cytophatic effect. Quantitative immunoblot analyses of membrane fractions from control and BFA-treated trophozoites utilizing monoclonal antibodies against the FN receptor and the lectin, revealed a marked decrease in both proteins, suggesting that the drug blocked their transport to the plasma membrane thereby impairing binding of the trophozoites to FN and to cell

surface galactose residues. These results agree with the previous report by Mann *et al*. (1991), who demonstrated that tunicamycin treatment of trophozoites inhibited Gal/GalNac lectin transport to the cell surface and adherence.

The FN receptor in *E. histolytica* has been characterized as a β1 integrin-like molecule, and other receptors for ECM proteins have been identified on the trophozoite surface (Rosales-Encina *et al*., 1992; Talamas-Rohana *et al*., 1998). Furthermore, a 37-kDa molecule associated with the cytoskeleton has been shown to be a fibronectin and laminin-binding molecule (Meza and Franco, 1988; Vazquez-Prado and Meza, 1992). Synergism between these molecules in the binding of trophozoites to the FN substrate, allowing degradation to occur even in the absence of a normal concentration of the b1 integrin-like FN receptor, would explain the observed FN degradation in the presence of BFA. Several cytoskeleton proteins have been identified in the cortical region of *E. histolytica* trophozoites. Actin is the major component but also present are myosins I and II, ABP-120, α-actinin, vinculin, PKC and ^{125}FAK (Arhets *et al*., 1995, Vazquez *et al*., 1995; Vargas *et al*., 1996). All of these proteins participate in one way or another in the dynamic association between the cytoskeleton and the plasma membrane that regulates membrane protein functions. Exocytosis is one of the cellular functions that require active participation of the cortical cytoskeleton (Verrey *et al*., 1995; Trifaro *et al*., 2000). We therefore analyzed this process in amoebae in the presence of BDM, a drug that specifically inhibits the ATPase activity of muscle myosin II and other non-muscle myosins, preventing contraction of actomyosin fibers without altering their arrangement (Higuchi and Takemori, 1989; Cramer and Mitchison, 1995; Castillo *et al*., 1998, 2002). The effect of BDM in processes that in *Entamoeba* are well characterized as myosin-dependent was tested and we found that this drug strongly inhibited cap formation, phagocytosis and pinocytosis. Furthermore, amoebae treated with BDM showed strong inhibition of the release of dextran-loaded vesicles, supporting our interpretation for myosin participation in the final step of exocytosis. As ameobae treated with BDM were also impaired in adhesion to FN and to cultured MDCK cells and BDM blocked the degradation of FN substrates, myosin-actin contractility seems to be required for release of the proteinases and for the proper function of the adhesion proteins. Quantitative analysis of plasma membrane fractions from trophozoites treated with BDM showed that the concentration of the FN receptor and the heavy subunit of the Gal/GalNac lectin were increased by approximately 25% compared to their concentration in untreated amoebae, further supporting that BDM does not affect the transit of vesicles to the membrane but that its main action is on the dynamic interaction between the actin-myosin network and the plasma membrane. As interactions among membrane proteins are modulated by cytoskeleton dynamics at the plasma membrane (Meza *et al*., 1982; Cantiello, 1995 and 1997; Castillo *et al*., 2002; Furuyashiki *et al*., 2002; Huang *et al*., 2002), it is entirely plausible that blocking actin–myosin interaction renders amoebae incapable of recycling membrane receptors and thereby impairs their function.

The present results show that E. *histolytica* trophozoites can transport proteins from the site of their synthesis through the cytoplasm utilizing at least two different pathways, one sensitive to BFA, with similarities to the classical route known for eukaryotic cells, notwithstanding the lack of typical secretory organelles, and another that follows an independent route that although could be constitutive, seems also to be regulated by external stimuli as has been shown for other cells (Andrews, 2000). Considering that secretory processes are important for the pathogenicity of this parasite and facilitate its dissemination through the host tissues, a clear understanding of them will help to identify checkpoints for blocking invasive behavior. In this sense, the present results provide interesting indications for possible targets.

Experimental procedures

Cell culture

Entamoeba histolytica HMI-IMSS trophozoites were grown axenically in TYI-S-33 medium (Diamond *et al*., 1978) at 37°C and harvested in the logarithmic phase of growth. Pellets were resuspended in 50 mM Tris, 150 mM NaCl, pH 7.4 (TBS), or TYI medium without or with serum for the experiments described below. MDCK cells (NBL-2) were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco cat. no. 12100–061, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum and 1% insulin at 37°C in a humid atmosphere containing 5% CO2.

Drug treatment

Brefeldin A (BFA) and 2,3-Butanedione monoxime (BDM) were purchased from Sigma Chemical Co. (St. Louis MO, USA). BFA was solubilized in dimethylsulfoxide (DMSO) and BDM in phosphate buffered saline pH, 7.4 (PBS); stock solutions were diluted to final concentrations of 100 μg ml^{-1} BFA, and 50 mM BDM. Amoebae were pretreated with the drugs for 2 h before the different assays. DMSO alone had not significant effect on the parameters measured. Viability of drug treated cells was assessed by staining with 10 mg ml^{-1} fluorescein diacetate and 30 μg ml^{-1} propidium iodide in TBS, pH 7.2 (Molecular Probes, Eugene, OR, USA). Under these conditions, viable cells are not stained with propidium iodide. Viability was greater than 95%.

Adhesion to fibronectin

Adhesion was determined by incubating untreated or drug-treated trophozoites on FN-covered 96-well plastic plates, as

previously described (Franco *et al.*, 1997). Adhesion to FN by control cells was standardized to 100%.

Cell damage

Confluent MDCK cell monolayers grown on 24-well culture plates were incubated with 2×10^5 trophozoites resuspended in TYI medium without serum in the absence or presence of BFA or BDM for 30–60 min at 37°C. After the incubation, the wells were rinsed repeatedly with TBS prewarmed to 37°C to remove dead cells. Live cells remaining in the wells were fixed with 3.7% formaldehyde and stained with methylene blue for 10 min Their amount was determined extracting the dye with 0.1 N HCl and its concentration measured at 595 nm using a Labsystems multiskan MS microplate reader. Damage inflicted by amebas untreated with BFA was normalized to 100%.

Fibronectin release assay

Trophozoites preincubated for 2 h in the absence or presence of BFA or BDM were layered on 96 well plates covered with ^{125}I-labeled FN and blocked with 0.1% BSA. Amoebae were incubated at 37°C with medium without serum containing the drugs. Forty μl aliquots of the culture medium were taken at the indicated times and radioactivity measured as previously indicated (Talamas-Rohana and Meza, 1988). Release values were calculated after substraction of non-specific release caused by the culture medium or by manipulation, that was never higher than 20%.

Pincytosis and Phagocytosis assays

Pinocytosis was evaluated by incubating 3×10^5 cells with 70 kDa fluorescein isothiocyanate-labeled dextran (FITC-dextran, 1.6 mg ml^{-1}) for 45 min at 37°C in TYI medium. The cells were washed with PBS and fixed with 3.7% formaldehyde for 20 min PBS-washed trophozoites were resuspended in 300 μl of PBS and the fluorescence quantified by fluorometry in a Becton-Dickinson cell sorter. The values obtained for cells not treated with BFA were normalized to 100%. Negative controls were established with parasites not exposed to FITC-dextran. Phagocytosis was evaluated utilizing 5×10^6 *E. coli* HB101 cells expressing green fluorescent protein (Clontech, Palo Alto, CA, USA). The bacteria were fed to trophozoites at a ratio of 5 : 1 and incubated for 45 min at 37°C. Trophozoites were washed twice with 5 mM sodium azide and 50 μg ml^{-1} gentamycin in PBS to remove surface-bound bacteria, then fixed with 3.7% formaldehyde for 20 min, washed with PBS, and resuspended in 300 μl of PBS. Fluorescence was quantified as above. The values obtained for trophozoites not treated with BFA were normalized to 100%. Negative controls were established using fixed parasites not exposed to fluorescent bacteria.

Eflux of Alexa 488 dextran

Trophozoites were loaded with 16 μg of Alexa 488 dextran (Molecular Probes) for 1 h in normal culture medium or in culture medium containing 50 mM BDM. After loading, the medium was changed to medium without the dextran for a chase of 2 h in the case of control cells and cells dextran-loaded in normal medium and chased in the presence of BDM. After the chase the cells were repeatedly washed with PBS and the concentration of Alexa 488 dextran inside the cells was determined by FACS analysis (emission peak 527 nm) utilizing 2×10^5 cells for each point.

Incorporation of ^{35}S-methionine into trophozoite proteins

Trophozoites (5×10^4) were metabolically labeled by addition of 100 mCi ml^{-1} of ^{35}S-methionine (1125 Ci μmol^{-1} in aqueous solution, ICN Cat. no. 51006) to the culture medium for 2 h at 37°C in absence or presence of BFA or BDM. After removal of the drugs and a chase of 2.5 h, 40 μl of culture medium were removed from the culture wells, and radioactivity incorporated into TCA-precipitable material determined using a beta liquid scintillator. Labeled cells were pelleted by centrifugation, washed with PBS and broken by freeze-thaw cycles to determine the radioactivity incorporated into TCA- precipitable material in the cell homogenate.

Isolation of plasma membranes and identification of membrane proteins

Trophozoites (2×10^7) were grown in absence or presence of BFA or BDM in TYI-S-33 medium for 2.5 h at 37°C. Plasma membranes were isolated from pelleted trophozoites as described by Aley *et al.* (1980). Aliquots containing 15 μg of protein were separated by electrophoresis in the discontinuous Laemmli system in 10% polyacrylamide gels and transferred to 0.1 μm nitrocellulose filters for immunoblot analysis (Towbin *et al.*, 1992). Monoclonal antibodies specific for the amoebic β1 integrin-like FN receptor, the heavy subunit (170KDa) of the Gal/GalNAc amoebic lectin and rat brain actin were used to identify the corresponding amoebic proteins. Positive bands were visualized with the ECL Western blot analysis system (Amersham Pharmacia RPN2106). Results were quantified by densitometry using Sigma Gel software 1.0.

Visualization of ER and Golgi proteins in trophozoites

Trophozoites grown on glass coverslips in TYI-S-33 medium were incubated in the absence or presence of BFA or BDM for 2 h at 37°C. Cells were rinsed with PBS, fixed with 3.7% paraformaldehyde warmed at 37°C and permeabilized with 0.1% Triton-X100. After extensive rinsing with PBS, the cells were incubated with the monoclonal antibodies (1:2 dilution for ERD2 and for PDI, and 1:100 dilution for actin). A secondary antibody tagged with Alexa R 488 (Molecular Probes) diluted 1:200 with PBS containing 1% FCS was layered on the cells and incubated for 30 min at 37°C. After thorough rinsing with PBS, coverslips were mounted with 100 μl of a gelvatol-p-phenylendiamine mixture, pH 8.5, for epifluorescence analysis using a standard Zeiss fluorescence microscope equipped with a Planapo 63X/1.4 objective.

Gelatin-substrate gels

Culture media from untreated trophozoite cultures and from drug-treated cells were recovered after 2 h. After protein determination

by Bradford, proteins were precipitated with cold acetone and denatured by boiling in electrophoresis sample buffer without β-mercaptoethanol. Aliquots containing 25 μg of protein were electrophoresed at 4°C under non-reducing conditions in 10% polyacrylamide gels copolymerized with 0.1% gelatin. Proteolytic activity was induced *in situ* by incubating the gels for 1 h at 37°C in the presence of thiol-activating buffer (0.1 M Tris pH, 7.5, 0.5% β-mercaptoethanol). Bands with proteolytic activity appear white after staining with Coomassie Blue.

Cap induction

Trophozoites grown on glass coverslips in TYI-S-33 medium were incubated in the absence or presence of BDM for 2 h at 37°C. Cells were rinsed with PBS and then incubated for 10 min with 40 μg ml^{-1} Con A/FITC in PBS in the absence or presence of BDM. After two PBS washes the cells were fixed for 30 min with 3.7% formaldehyde. After thorough rinsing with PBS, coverslips were mounted with 100 μl of a gelvatol-p-phenylendiamine mixture, pH 8.5, for epifluorescence analysis.

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