J. Euk. Microbiol., 41(4), 1994, pp. 360–365

Actin mRNA Levels and Actin Synthesis During the Encystation of *Entamoeba invadens*

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ABSTRACT. Parasitic amebas propagate among hosts through cysts, the resistant forms in their life cycle. In spite of their key role in infection, little is known about the encystation process and the mechanisms involved in reaching this stage. Two features drastically affected by encystation are motility and cell shape, both of which are determined by the cytoskeleton, composed mainly of actin in these organisms. Therefore, we studied the occurrence and relative levels of actin and actin synthesis during encystation of *Entamoeba invadens*. Using a cDNA actin probe obtained from a library of *E. histolytica* and a monoclonal antibody against actin, we found that, while the total actin levels sharply decrease as encystation proceeds, the levels of actin mRNA are reduced only in mature cysts. Moreover, actin synthesis does not take place in precysts and the later stages of cyst formation. In contrast, the levels of other proteins remain stable in trophozoites, precysts and cysts, and stage specific peptides are actively synthesized in precysts. The results indicate that encystation is accompanied by a preferential down-regulation of actin synthesis and a decrease in actin levels. The reorganization of the cytoskeleton occurring as trophozoites transform into round, quiescent cells, could be a regulatory factor in the observed changes.

Supplementary key words. Cell shape, cytoskeleton, gene expression, parasites, regulation.

DURING their life cycle, parasitic amebas go through marked changes in shape and motility. From active trophic forms capable of rapid ameboid locomotion, phagocytosis and endocytosis, they change to immobile round cells—precysts and cysts—that gradually become enveloped by an impermeable chitin wall [17, 18]. Therefore, the cellular and biochemical processes that control motility in trophozoites would be expected to undergo considerable modifications during encystation.

Actin is the most prominent component of the cytoskeleton in amebas. A key participant in ameboid movement, it is probably also involved in many of the processes taking place during encystation. Actin has been visualized in moving trophozoites of *E. invadens* [5] and fully characterized in *E. histolytica* trophozoites, where it constitutes 15–20% of the total protein [19]. It is directly involved in the adhesion to specific substrates and targets [27], release of lytic factors [24], phagocytosis [6], cell shape and all the cell movements that facilitate invasion of host tissues [20]. Apparently only one isoform of actin is expressed in trophozoites, but at least four actin genes have been identified in the genome of *E. histolytica* strains [12] and as many as seven in *E. invadens* [Manning-Cela, R. 1990 M.Sc. Thesis CINVESTAV, México]. The role of actin in the motility and pleiomorphism of trophozoites has been inferred by fluorescence microscopy observations using Rhodamine-phalloidin and an actin antibody [5], and from experiments using cytochalasin D, which by disorganizing the actin cytoskeleton renders amebas incapable of adhesion and phagocytosis [20, 24]. Furthermore, studies with motility mutants of *E. histolytica* have shown alterations in the actin distribution concomitant with attenuated virulence [10].

The information available about actin in the trophic stage of *E. invadens* and *E. histolytica* contrasts with the lack of data about its presence and its role during encystation. A partial explanation for this scarcity of data is the difficulty in inducing reproducible in vitro encystation of trophozoites in axenic culture—in particular for the species that are parasites of humans—as well as the high resistance and impermeability of the cyst wall which hampers the preparation of samples and subsequent biochemical analyses. On the other hand, *E. invadens* a parasite of reptiles, offers the advantage of encystment in axenic conditions when subjected to environmental stress, such as depletion of carbohydrates and changes in osmolarity [25].

We used a cDNA actin probe obtained from an *E. histolytica* expression library, and a monoclonal antibody against actin, to measure actin mRNA and actin levels as well as actin de novo synthesis during the encystation of *E. invadens*. To our knowledge, this is the first report on the presence of actin in *Entamoeba* precysts and cysts, and the first attempt to establish a correlation between actin expression and the changes in cell shape and motility that accompany specific stages in the life cycle of parasitic amebas.

MATERIALS AND METHODS

Entamoeba cultures. Trophozoites were grown in the axenic medium TYI-S-33 as described by Diamond [11]. *E. histolytica* HM-1:IMSS (originally isolated and typified at Centro Médico Nacional, IMSS, México) was grown at 37° C and *E. invadens* IP-1 (ATCC No. 30994) at 26° C. Amebas were pelleted by centrifugation at 400 *g* for 4 min and washed twice with phosphate buffered saline, pH 7.5 (PBS).

Preparation of precysts and cysts from *E. invadens*. The method used to encyst trophozoites was basically the one reported by Rengpien & Bailey [25]. The precysts and cysts, harvested at 42 and 72 h after transfer of trophozoites to encystation medium, were separated in a discontinuous 10–100% Percoll density gradient according to Avron et al. [3].

RNA isolation. Frozen trophozoite pellets were thawed by suspension and gentle swirling in 3 equivalent volumes of 4 M guanidinium isothiocyanate (Fluka, Buchs, Switzerland), 50 mM Tris pH 7.6, 10 mM EDTA, 1.4 mM beta-mercaptoethanol, 2% Sarkosyl at 4° C. After centrifugation at 7,000 *g* at 4° C the supernatant was layered on a 1.2 ml cushion of 5.7 M CsCl prepared in 0.1 M EDTA pH 7.5 and centrifuged at 147,215 *g* for 17 h at 16° C (SW rotor 50.1). The pellets were dissolved in 10 mM Tris pH 7.6, 5 mM EDTA, 1% SDS (TES) and RNA precipitated by addition of 0.3 M sodium acetate and 2.5 volumes of ethanol. Pellets were resuspended in diethyl-pyrocarbonated treated water; RNA was assayed by agarose/formaldehyde gel electrophoresis. To obtain RNA from precysts and cysts, these were broken with glass beads by vigorous vortexing in 3 volumes of 4 M guanidinium isothiocyanate, 50 mM Tris pH 7.6, 1 mM beta-mercaptoethanol, 2% Sarkosyl and 1 volume of saturated phenol for 1 h at 4° C. From here on, the preparation was the same as for trophozoites.

Lambda-Ethact-cD1 DNA preparation. The actin clone Ethact-cD1 was isolated from a cDNA library prepared in lambda-gt11 [12]. The phage DNA was purified as reported by Maniatis et al. [26] and the insert obtained by digestion of the DNA with EcoR1, which generates 2 fragments of 0.4 kbp and 0.7 kbp. The two fragments were recovered by electrophoresis from preparative agarose gels into DEAE papers [15]. DNA were

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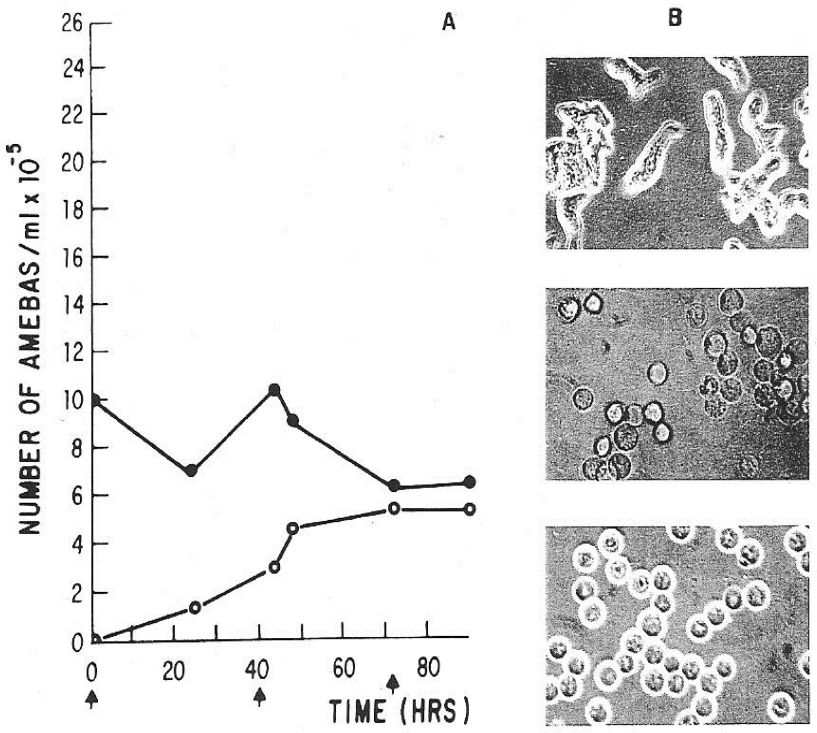

Fig. 1. Encystation of *E. invadens*. **A.** Encystation curve showing the total number of cells in culture (●——●) and the total number of cells resistant to 1% sarkosyl (O——O). Arrows indicate the times at which the cells were harvested for the best yield of trophozoites, precysts and cysts. **B.** Phase-contrast photomicrographs depict the appearance of the cells at the above indicated stages starting from the top.

recovered from the papers and labeled using the Amersham Multiprime DNA labelling kit (catalog No. RPN 1,601Y), with ^{32}P-deoxycytidine (6,000 Ci/mmol).

rDNA probe. The ribosomal 26S rDNA 3′ probe was isolated from a genomic library of *E. histolytica* HM-1:IMSS prepared in Bluescript plasmid (Edman et al., unpubl. data). This clone contains a fragment of 1.6 Kb in the restriction site EcoR1-HindIII located at the 3′ end of the 26S ribosomal cistron sequences. The plasmid DNA was purified as reported by Sambrook et al. [26] and labeled as reported above.

Dots and transfer blots. RNA blots were prepared by standard methods [13, 26]. Hybridizations were performed at 42° C with 50% formamide. Washings were done either at low stringency conditions (6 × SSC/4° C) or at high stringency (0.1 × SSC, 0.1% SDS/55° C). Hybridizations were performed in parallel dots and also using the same blot and rehybridizing the rRNA probe after removing the actin probes [26]. The relative concentration of actin mRNA in relation to total RNA in each sample was quantitated from three separate experiments using a scanning densitometer (DU-7 spectrophotometer, Beckman) coupled to an integrator.

Immunoblot of cell extracts. Pellets of separated trophozoites, precysts and cysts, were suspended in PBS containing 10 mM para-hydroxymercuribenzoate (PHMB) and boiled for 5 min. Precysts and cysts were broken with glass beads in PBS/PHMB before boiling. Aliquots containing 100 μg of protein were run in the discontinuous Laemmli system in 10% polyacrylamide and transferred to 0.1 μm nitrocellulose filters for immunoblot analyses [28]. An anti-actin antibody from a clone producing IgG against the gamma isoform of rat brain actin, was used. Positive bands were visualized with 2-4-dichloro-1-naphtol and scanned in a densitometer as indicated above. Actin quantification corresponds to the average of three separate experiments. Polyclonal antibodies against the 96 KDa region peptides were obtained by immunizing mice with the 96 KDa band cut from SDS-polyacrylamide gels of amebic extracts.

Immunoprecipitation of ^{35}S-labeled actin. Trophozoites, precysts and cysts were metabolically labeled with ^{35}S-methionine (1,125 Ci/mmol in aqueous solution Cat. No. 51006 ICN). Trophozoites were transferred to encystation medium and 35 μCi of labeled methionine were added per ml of medium. Labeled cells were harvested at the times corresponding to trophozoite (5 h), precyst (42 h) and cyst stages (72 h). To cells already in encystation medium, the label was added at precyst stage (42 h) and cyst stage (67 h) as indicated above, and labeled cysts were recovered at 72 h of culture. Trophozoites were suspended in 100 μl of PBS containing 10 mM PHMB and broken by freeze-thaw cycles. Precysts and cysts were broken with glass beads.

The amount of radioactivity incorporated was determined in 2 μl aliquots after TCA precipitation. Aliquots of 10 μl from each sample were incubated for 10 h at 4° C with one volume of the anti-actin antibody. After incubation with the second antibody for 1 h at 26° C, protein A beads resuspended in 15 mM Tris pH 7.5, 5 mM EDTA, 2 mM EGTA, 1% triton X-100, 0.1% SDS, 1% deoxycholic acid, 0.5% BSA, 120 mM NaCl, 25 mM KCl, 10 mM PHMB (AA buffer) were used to precipitate the antibody-actin complexes. Precipitated samples were washed with AA buffer and solubilized by boiling in electrophoresis sample buffer. Results represent one of three separate experiments.

RESULTS

Encystation of trophozoites. Trophozoites were grown for seven days in Diamond's TYI-S-33 medium and then thoroughly washed with PBS and suspended in the hyposmotic medium to induce encystation. The encystation curve in Fig. 1 shows the growth of the cells and their differentiation into cysts. The transformation was monitored by the resistance of the cells to 1% sarkosyl and by staining with calcofluor, both indicators of the presence of a chitin wall [2]. The changes in shape accompanying the resistance to detergent were visualized by phase-contrast microscopy of the cells separated in Percoll gradients

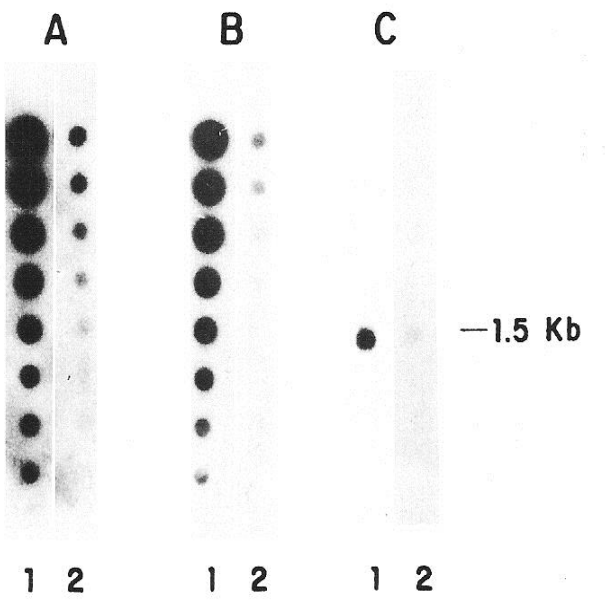

Fig. 2. Actin mRNA in *E. invadens*. Dot blot hybridization of *E. histolytica* and *E. invadens* total RNA with the 0.7 Kbp actin probe. Two-fold serial dilutions of an initial RNA amount of 7.5 μg were spotted. **A**. relaxed conditions. **B**. stringent conditions. **C**. Northern blot hybridization. Lane 1, *E. histolytica*; lane 2, *E. invadens*.

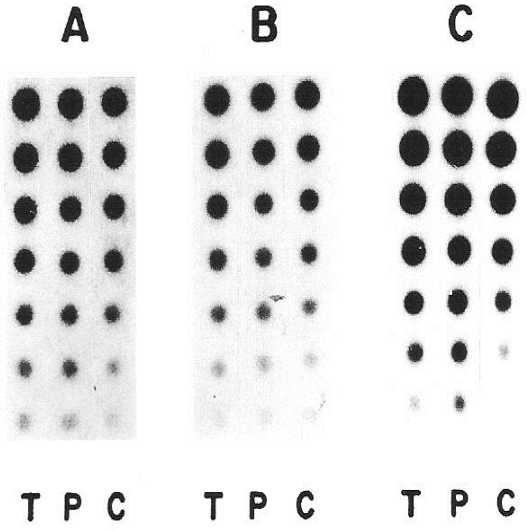

Fig. 3. Levels of actin mRNA and rRNA during encystation. **A**. Dot blot hybridization of two-fold serial dilutions of total RNA from *E. invadens* with the 0.7 Kbp fragment of the actin probe. **B**. Dot blot hybridization of two-fold serial dilutions of total RNA from *E. invadens* with total DNA from the same cells. **C**. Dot blot hybridization of two-fold serial dilutions of total RNA from *E. invadens* with the 26S rDNA 3′ probe. The total RNA spotted on the first dot corresponded to 7.5 μg. T, trophozoites; P, precysts; C, cysts.

(photomicrographs). Precysts banding at 40–50% Percoll and cysts banding at 30% Percoll were separated from trophozoites, which band diffusely from 70% Percoll to the bottom of the tube. The separated cells were then counted to determine the percentage of encystation. The average yield of cysts in more than 10 different experiments was 80%.

Actin mRNA in *E. invadens*. To test the possibility of using a heterologous actin probe, total RNA was obtained from *E. invadens* and *E. histolytica* trophozoites in the logarithmic phase of growth. Fifty-microliter aliquots of serial 2-fold dilutions of the initial RNA concentration (0.15 mg/ml) were blotted onto nitrocellulose membranes. The first dot contained 7.5 μg of RNA. The 3′ end (0.7 kbp) and the 5′ end (0.4 kbp) fragments of the Ehact-cD1 actin probe from *E. histolytica* were then hybridized to the blots. Figure 2 shows the results obtained with the 0.7 kbp fragment hybridized under relaxed conditions (A) and under stringent conditions (B). A positive signal is clear for the homologous *E. histolytica* RNA all the way to dilutions of 0.05 μg (lane 1, A and B), while for the heterologus *E. invadens* RNA a clear signal is seen up to 0.9 μg under relaxed conditions, and up to 3.7 μg for the stringent conditions (lane 2, A and B respectively). Panel C shows the northern blots of the same two RNA hybridized with the 0.7 kbp fragment under stringent conditions. The single positive band in *E. histolytica* corresponds to the reported 1.5 Kb actin mRNA size [12]. The band of similar size seen for *E. invadens* suggests that, in this ameba, actin mRNA could also correspond to a single molecular species. Identical results were obtained using the 0.4 kbp fragment of the actin probe in dot as well as in northern blots (data not shown). The hybridization results using stringent or relaxed conditions indicated partial homology of the actin mRNA sequences between the two *Entamoeba* species, in agreement with recent reports using rRNA probes that reveal that *E. invadens* is a distant relative of *E. histolytica* [8]. Therefore, in the experiments described below relaxed conditions were used for the hybridizations.

Levels of actin mRNA during encystation. Total RNA was extracted from Percoll-gradient-separated trophozoites, precysts and cysts harvested at the times indicated by the arrows in the encystation curve (Fig. 1). The actin probes of 0.4 and 0.7 Kbp were hybridized under relaxed conditions to serial 2-fold dilutions of the RNA samples in dot blots. Hybridization with the 0.7 kbp fragment is shown in Fig. 3A. Densitometric scanning of the radioactivity spots (considering the linear part of the curve) indicated that the relative levels of actin mRNA in trophozoites and precysts remain very similar, while a 50% decrease was measured in the cysts. To test if actin mRNA was the only RNA which levels varied during encystation, we hybridized *E. invadens* RNA with the 26S rRNA 3′ probe. The results shown in Fig. 3 (panel C) indicate that the levels of rRNA are also 50% lower in cysts than in trophozoites and 50% higher in precysts, suggesting fluctuations in the levels of rRNA during encystation. These fluctuations might be explained by the rearrangement of ribosomes into chromatoid bodies and the higher lability of rRNA and ribosomes reported during the aging of amebic cultures and also in cysts of *E. invadens* [1, 21]. As a control for the amount of RNA blotted on the dots, ^{32}P-labeled DNA from *E. invadens* trophozoites was hybridized to RNA blots made in parallel with the blots hybridized with the actin and rRNA probes. As shown in Fig. 3B, the total amount of RNA in each one of the dilutions is comparable for the three stages of encystation, so the hybridization signals reflect real differences in actin mRNA and rRNA levels in the later stages of encystation.

Levels of actin during encystation. The actin band was identified by immunoblot of cell extracts using the actin monoclonal antibody (panel A in Fig. 4). The specificity of the antibody is shown in a blot of partially purified skeletal muscle actin (M). Results obtained from three separate experiments after scanning the blots and measuring the areas under the curves, indicated that the levels of actin decreased to 48% in precysts as compared to the levels found in trophozoites (lanes 1 and 2) and as much as 80% in cysts (lane 3). To rule out the possibility of actin degradation during the inherent difficulties in breaking the precyst and, particularly, the cyst wall, the levels of two amebic peptides in the 96-kDa region—known to be prominent in trophozoite extracts—were also analyzed by immunoblot (panel

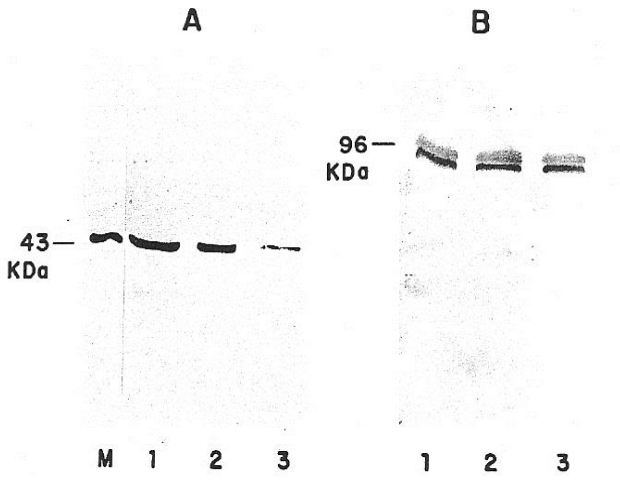

Fig. 4. Relative actin levels during encystation. Total cell extracts from 1. trophozoites, 2. precysts and 3. cysts of *E. invadens* were separated by SDS-PAGE and transferred for immunoblot. **M**, partially purified skeletal muscle actin, **A**, anti-actin monoclonal antibody, **B**, polyclonal antibody against the two amebic peptides of 96-KDa.

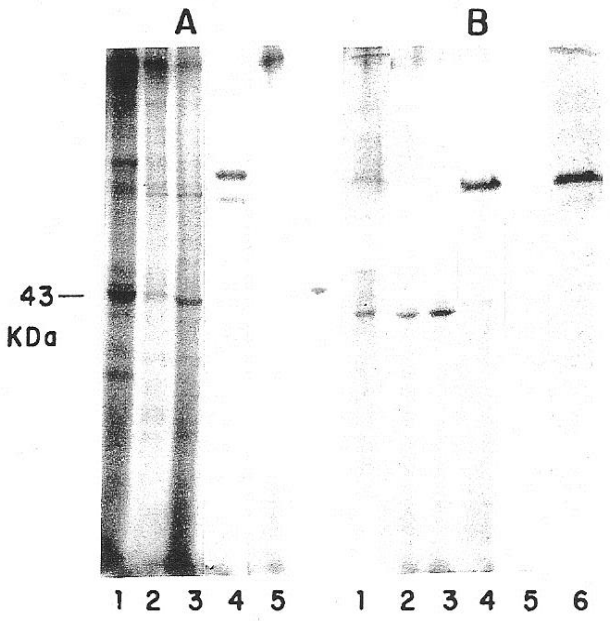

Fig. 5. ^{35}S-methionine incorporation into cell proteins and immunoprecipitation of labeled actin. **A**. Fluorograph of labeled proteins from *E. invadens* extracts separated in 10% gels by SDS-PAGE. Lanes 1, 2 and 3 show the pattern of trophozoites, precysts and cysts labeled during trophozoite stage. Lane 4 shows the pattern of proteins in cysts labeled at precyst stage. Lane 5 shows the pattern of proteins in cysts labeled at cyst stage. **B**. Immunoprecipitated actin from cells labeled as indicated in A, separated by SDS-PAGE. Lane 1–3, immunoprecipitated samples from trophozoites, precysts and cysts labeled during the trophozoite stage. Lane 4, immunoprecipitated sample from cysts labeled at precyst stage. Lane 5, immunoprecipitated sample from cysts labeled at cyst stage. Lane 6, same as lane 4 but without the precipitation steps with antibody and protein A.

B). Contrary to what is seen for actin, the levels of these peptides remained constant during encystation.

Actin synthesis during encystation. The results above suggested that actin is differentially synthesized or differentially degraded during encystation. To distinguish between these two possibilities, trophozoites, precysts and cysts were metabolically labeled with ^{35}S-methionine and the incorporated radioactivity was measured after TCA precipitation of cell extracts from each stage. As seen in Table 1, incorporation of radioactive precursors into TCA precipitable material is actively taking place in trophozoites, while the incorporation of label into precyst proteins decreased to 20% of that measured in trophozoites. Cysts incorporate only 10% of the label taken by trophozoites. Figure 5A shows an autoradiograph with the pattern of labeled proteins in trophozoites, precysts and cysts. When the label is added during the trophozoite stage, several labeled proteins appear (lanes 1–3). A band of 43-KDa is present in all the stages, whereas two peptides of 35 and 90 KDa are present only in trophozoites. It is also clear that less labeled proteins are present in precysts and cysts, although they could have been synthesized during the trophozoite stage. Labeling during the precyst stage (lane 4) identifies four peptides of 195, 150, 80, and 75 KDa which are specifically synthesized in this stage. Some of these peptides could correspond to the proteins reported as produced during cyst formation [4, 9], none of which is actin. Radioactive label added to cysts is not incorporated into detectable protein bands (lane 5).

Table 1. ^{35}S-methionine incorporation during encystation.

Stage of encystation in which label was added	Stage of encystation in which label was analyzed[a]	Cpm/10^6 cells[b]
T	T	377,200
P	C	75,840
C	C	31,160

T, Trophozoite; P, Precyst; C, Cyst.
[a] Analyzed by TCA precipitation of cell extracts.
[b] Average of two separate experiments.

These results indicate that protein synthesis takes place in trophozoites and precysts but not in cysts, and that actin is therefore synthesized during the trophozoite stage, although at this point we can not rule out the possibility that cysts can not incorporate precursor because of the relative impermeability of the chitin wall. The results in Fig. 5B, showing that radioactive actin is immunoprecipitated by the actin antibody in precysts and cysts only if these were labeled at trophozoite stage (lanes 1–3), confirm that actin is synthesized in trophozoites but not in precysts where radioactive actin is not precipitated (lane 4). The labeled peptide corresponds to the 80-KDa cyst wall protein that as part of the particulate fraction settles down by gravity. The same band is seen in lane 6, which corresponds to cyst wall pellets labeled during precyst stage and to which no antibody nor protein A were added. Lane 5 contains proteins from cysts labeled at cyst stage and incubated with anti-actin antibody. No radioactive bands were detected.

DISCUSSION

The differentiation of parasitic amebas into cysts remains one of the least known aspects in the biology of these organisms. Information on any of the biochemical and molecular processes leading to encystation would be important not only to understand morphogenesis, but also to design means by which the life cycle of the parasite could be interrupted. As motility and cell shape are greatly modified during encystation and they directly depend on the actin cytoskeleton, we studied the levels of actin and the regulation of actin mRNA and actin synthesis.

Our results show that while the levels of actin mRNA—relative to total RNA—are maintained without significant change

in trophozoites and precysts (Fig. 3A), the levels of total actin—relative to total protein—in precysts decrease to 48% (Fig. 4A). At the same time, the amount of active actin synthesis taking place in trophozoites is drastically reduced in precysts while de novo synthesis of stage specific peptides is otherwise triggered (Fig. 5). In cysts, the levels of actin mRNA decreased to 50% of those found in trophozoites and precysts (Fig. 3A) and there was no apparent synthesis of actin (Fig. 5). Furthermore, a marked decrease (80%) in the levels of total actin was observed in cysts (Fig. 4). Although there is a general decrease of protein synthesis in precysts, as indicated by the incorporation of radioactive precursors (Table 1), and the cysts may shut down the protein synthesis machinery as they reach a dormant state, not all the proteins are degraded at the same rate. The relative levels of two peptides in the region of 96-KDa, against which polyclonal antibodies were produced, remained constant during encystation whereas the relative levels of actin clearly decreased during cyst formation (Fig. 4A, B).

In many eukaryotic systems actin expression is linked to the organization of actin microfilaments and cell morphology. For example, disruption of the microfilament network in differentiating muscle, with the concomitant changes in cellular shape, downregulates actin mRNA levels [22]. Since the translation of mRNA in the cytoplasm is associated with cytoskeleton organization [23], it has been suggested that, in addition to its role in maintaining cell morphology and plasticity, the cytoskeletal network might have a major regulatory role in gene expression [7]. In the case of *Entamoeba,* where dramatic changes in cell shape and motility occur as a result of encystation, it is conceivable that the cytoskeleton, by regulating the morphology and activity of the amebas, also regulates actin synthesis and actin levels. In support of our conclusions, it has been shown that during encystation of the free-living *Acanthamoeba castellanii* a gradual but small decrease in actin mRNA is initially accompanied by a 2-fold reduction of actin synthesis, which in later stages increases to 10-fold [14]. In this case, as in *Entamoeba,* the sharp decrease in actin synthesis can not be completely explained by the lower levels of actin mRNA. Other regulatory events, such as compartmentalization of the mRNA and its redistribution together with ribosomes, may act in concert with the actin cytoskeleton [16]. While there is no evidence that any of the above processes occur in *Entamoeba,* it is known that during cyst formation ribosomes aggregate into dense structures called chromatoid bodies, which renders them at least partially non-functional [1, 21]. This rearrangement may be related to the fluctuation in rRNA levels detected with the 26S rDNA 3′ probe. On the other hand, the alterations of the cytoskeleton and the disruption of the dynamic equilibrium of actin filaments, preceeding or accompanying encystation, could lead to the protein synthesis shut down and the observed progressive decrease in total actin levels.

Unfortunately, the measurement of polymerized vs. soluble actin in precysts and cysts is not feasible because the chitin envelope interferes with the penetration of actin markers. Furthermore, our ability to study the transformation of cysts into motile ameboid trophozoites—a step that would require reorganization of actin filaments to trigger actin mRNA and actin synthesis, providing direct proof of transcriptional control—is also hampered by the current lack of experimental conditions able to induce efficient excystation.

Our results suggest that in trophozoites and cells at the early stages of encystation, which are still very active, mobile and maintain an organized dynamic cytoskeleton, actin synthesis is mainly controlled by the levels of actin mRNA. At the later precyst and cyst stages, when actin synthesis and actin levels decrease more than mRNA levels, other control mechanisms, presumably at the level of protein translation and possibly linked to the disorganization of the cytoskeleton, regulate the synthesis and half-life of actin.

ACKNOWLEDGMENTS

We are grateful to A. Boyzo for keeping the *E. invadens* and *E. histolytica* cultures. R.M.C. was supported by a predoctoral fellowship from CONACyT, Mexico. Research partially supported by grants from CONACyT (904013) and the Mac Arthur Foundation.

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Received 8-26-93, 11-29-93, 1-14-94; accepted 2-4-94