Cloning and Molecular Characterization of a cDNA Clone Coding for *Trichomonas vaginalis* Alpha-Actinin and Intracellular Localization of the Protein

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We have identified and sequenced a cDNA clone coding for *Trichomonas vaginalis* alpha-actinin. Analysis of the obtained sequence revealed that the 2,857-nucleotide-long cDNA contained an open reading frame encoding 849 amino acids which showed consistent homology with alpha-actinins of different species. Such homology was particularly significant in regions which have been reported to represent the actin-binding and Ca²⁺-binding domains in other alpha-actinins. The deduced protein was also characterized by the presence of a divergent central region thought to play a role in its high immunogenicity. A study of protein localization performed by immunofluorescence revealed that the protein is diffusely distributed throughout the *T. vaginalis* cytoplasm when the cell is pear shaped. When parasites adhere and transform into the amoeboid morphology, the protein is located only in areas close to the cytoplasmic membrane and colocalizes with actin. Concomitantly with transformation into the amoeboid morphology, alpha-actinin mRNA expression is upregulated.

The flagellated protozoan parasite *Trichomonas vaginalis* is the etiologic agent of one of the most widespread sexually transmitted diseases worldwide. The main pathological manifestations of a trichomonad infection in women are abdominal pain, itching, and presence of a foul-smelling discharge with abundant leukocytes (19), while in men the infection is mostly asymptomatic, although it can sometimes lead to urethritis, prostatitis, and epididymitis (24). The infection recently has been associated with severe complications, such as infertility (28), enhanced predisposition to neoplastic transformation in epithelial cells (18). Several in vitro studies have reported that adherence of the parasite to the target cell is essential for the maintenance of infection and for cytopathogenicity (2, 25). Using erythrocytes as a target cell model, we recently demonstrated that the cytopathic effect of *T. vaginalis* is mediated by pH-dependent perforins (1, 14) and by a contact-dependent disruption of the cortical cytoskeleton (13). The results obtained in our studies highlighted the importance of an intimate association between the parasite and the target cell membranes. Contact of *T. vaginalis* with epithelial cells induces significant changes in parasite morphology. The ability of *T. vaginalis* to change from ellipsoidal to amoeboid morphology when it encounters the target cell seems to represent a virulence trait (5). Moreover, the motility and plasticity of the parasite are important for pathogenic activity (16). The ability to undergo a morphological transformation upon contact with the target cell requires the presence of a complex and ductile cytoskeletal structure, which needs to be finely tuned and able to respond promptly to external stimuli. The parasite cytoskeleton has been the subject of different studies (7, 22), but the relationships between the molecular components of this structure and the morphological changes that occur during parasitism have never been studied. It was recently discovered that *T. vaginalis* and other protozoan parasites possess complicated pathways and complex regulation mechanisms which were believed to exist only in higher eukaryotes. For example, the presence in *T. vaginalis* of calmodulin and of E2 ubiquitin-conjugating enzyme was recently reported (23).

In this study, we report the isolation, nucleotide sequencing, and characterization of a cDNA coding for *T. vaginalis* alpha-actinin. Alpha-actinin is an actin-binding, Ca²⁺-regulated protein involved in actin cross-linking. It is widely distributed among different cellular types. The actin-binding protein family plays a fundamental role in motion and morphological changes, since motion is a consequence of the cellular redistribution of actin.

The alpha-actinin cDNA sequence was characterized at the nucleotide and amino acid levels, revealing several interesting features of the molecule. In addition, a study of the cytoplasmic localization in pear-shaped and amoebic parasites was performed.

**MATERIALS AND METHODS**

**Strains and culture conditions.** Thirty *T. vaginalis* isolates were obtained from vaginal specimens of women affected by trichomoniases in Italy and Mozambique. Organisms were axenically grown in Diamond’s Tryptose-yeast medium (10). For experiments in which a single strain was used, we chose isolate SS-22, already used in all our previous works as a standard *T. vaginalis* strain.

*Giardia lamblia* was kindly provided by L. Gradoni, Istituto Superiore di Sanità, Rome, Italy; *Acanthamoeba castellanii* was a gift from P. Varaldo, University of Ancona, Ancona, Italy; and *Leishmania major* was kindly provided by C. Bordier, University of Lausanne, Lausanne, Switzerland. *Entamoeba histolytica* HK-9 was obtained from the American Type Culture Collection. Protozoa

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were cultured either in media suggested by the American Type Culture Collection or by standard procedures (11, 36). One microliter of DNA from each isolate was submitted to PCR to assess the presence of the alpha-actinin gene. Primers TV44 (5'-AGGAGTGTGGTATGTTG-3') and TV54 (5'-TCGCTCTTCTGCTATT3') were synthesized by use of a Gene Assembler Plus (Pharmacia, Uppsala, Sweden). Amplification was performed by the Sanger dideoxy chain termination method with a Sequenase 2.0 (United States Biochemical Corp., Cleveland, Ohio) and T7 and T3 primers, recognizing specific regions in the multiple cloning sites, were used. The obtained, quantified RNA was electrophoresed in 1% agarose–2 M formamide, and the RNA bands of 25 kDa were excised. The recombinant plasmids were transferred to Hybond-N membranes (Amersham). The DNA probe used to detect alpha-actinin mRNA was prepared by PCR with primers TV44 and TV54, while the resulting PCR products were electrophoresed in a 1% agarose gel and visualized with a UV transilluminator after ethidium bromide staining. RESULTS

Isolation of cDNA clones and expression of the recombinant gene. Previous studies performed on T. vaginalis adhesion proteins led us to the observation that erythrocytes lysed by the microorganism displayed both the major adhesion proteins already described in previous studies (4, 15) and a 115-kDa trichomonad protein that was present in small amounts. The protozoan protein was present on the host cell surface after completion of lysis by live T. vaginalis or after incubation of target cells with protozoan lysates. In order to identify and characterize the 115-kDa T. vaginalis protein, specific antibodies were eluted from a rabbit hyperimmune anti-T. vaginalis serum. The eluted antibodies, which were monospecific and did not show cross-reactivity with target cell antigens, were used to screen a cDNA expression library. The screening led to the isolation of several positive phage clones. One of the clones was chosen, and the pBluescript (SK)–plasmid was excised and used to transform competent E. coli cells. The transformed cells produced a recombinant protein of about 110 kDa, suggesting that the cDNA clone coded for almost the entire sequence. The recombinant protein was readily recognized by antibodies eluted from the 115-kDa native protein bound to erythrocytes (data not shown). More-
over, antibodies eluted from the recombinant plaques recognized the native protein.

DNA sequencing and characterization. Only one open reading frame was predicted for the cloned cDNA, spanning nucleotides 1 to 2548. The derived amino acid sequence is represented in Fig. 1A. A scan of SWISS-PROT and GenBank revealed significant similarity of the amino acid sequence with alpha-actinins from other species. The amino acid sequence showed 26% identity with Dictyostelium discoideum and Drosophila melanogaster alpha-actinins (45 and 43%, respectively, when conservative changes were considered).

Identity with alpha-actinin was considerably higher in some areas. For the nucleotide sequence, in the 3’ region identity was as high as 54.15% on 698 nucleotides with human alpha-actinin (3), 55.35% on 598 nucleotides with chick alpha-actinin, and 54.97% on 553 nucleotides with rabbit alpha-actinin. Homology was also found with spectrin, being higher for nucleotides 400 to 700, where the sequence showed 60.5% identity with mouse beta-spectrin. The 5’ region of the sequence displayed very high identity with different calmodulin genes. Nucleotides 2357 to 2522 showed 72 to 64% identity with genes for calmodulins of different species. Consistent with the early divergence of this protozoan parasite, the highest identity was observed with genes for calmodulins of several plants, such as wheat, barley, and maize.

The N-terminal region of the translated sequence was the area where identity with alpha-actinin was higher. In the region spanning amino acids 93 to 197, the sequence was 49% identical to D. discoideum alpha-actinin. Identity was increased to 78% when conservative changes were considered. This region contains the domain responsible for the binding of actin in actin-binding proteins. Figure 1B shows the actin-binding domains of other alpha-actinins and a consensus sequence for the actin-binding domain. Moreover, comparison of the T. vaginalis alpha-actinin cDNA translated sequence revealed identity with other proteins belonging to the actin-binding protein family, such as spectrin, dystrophin, and myosin. Amino acids from positions 4 to 386, for example, displayed 29% identity (49% similarity) to the D. melanogaster spectrin b-chain. Analysis of the protein sequence confirmed the high homology with calmodulin observed in the 5’ region of the nucleotide sequence.

The C-terminal region of the protein (amino acids 803 to 833) contains a Ca\(^{2+}\)-binding domain known as the EF-hand. Figure 1C shows the alignment of this domain with the EF-hands of other known alpha-actinins and with the consensus sequence given by Tufty and Kretsinger (33) for chick alpha-actinin. The T. vaginalis alpha-actinin EF-hand appears to be functional, since it is similar to the consensus sequence. Intra-cellular rearrangement of the protein could therefore be mediated by signalling mechanisms involving Ca\(^{2+}\) ions.

The central region of the protein (amino acids 387 to 650), as shown in Fig. 2, showed less significant homology with alpha-actinin and other cytoskeletal proteins. Analysis of the amino acid sequence for antigenic peptides revealed that some of the predicted areas of highest antigenicity were located in this region (amino acids 682 to 687, 409 to 414, and 377 to 382). The diversity of this region from human alpha-actinin might be interesting in terms of the immunogenicity of the protein.

As expected for a structural protein, the gene coding for the molecule was present in all 30 T. vaginalis strains tested, independent of the geographical origins of the patients (data not shown).

Specificity of the T. vaginalis alpha-actinin immunogenic determinants. A high degree of identity was observed between the trichomonad alpha-actinin and alpha-actinins from other species. However, the sequence analysis revealed the presence of a divergent peptide region in the central portion of the protein. Computer-assisted analysis revealed that the antigenic determinants most likely were located in this region. Since high identity with other alpha-actinins is present throughout the other regions of the T. vaginalis protein, we wanted to test whether antibodies raised against the trichomonad protein reacted with other alpha-actinins of different origins. Since the main targets of T. vaginalis in vivo are human epithelial cells, an immunoblot study was performed by probing protein ex-
tracts obtained from these cells with anti–trichomonad alpha-actinin antibodies. The results obtained revealed that there was no cross-reactivity; monospecific antibodies directed against *T. vaginalis* alpha-actinin did not recognize the human form of the protein. In order to assess cross-reactivity with other protozoan actin-binding proteins, we tested protein extracts obtained from other protozoan parasites, *G. lamblia*, *E. histolytica*, *A. castellanii*, and *L. major* total proteins were tested. No cross-reactivity was observed with any of them. The absence of cross-reactivity between parasite and host alpha-actinins was also observed by immunofluorescence. Figure 3 shows the alpha-actinin fluorescence in *T. vaginalis* parasites coincubated with epithelial cells. The localized peripheral fluorescence was clearly visible, while the host cells showed no fluorescence. The lack of fluorescence of epithelial cells confirmed that there was no immunological cross-reactivity between parasite and host proteins, as already shown by the immunoblot studies. Therefore, we hypothesize that anti–trichomonad alpha-actinin antibodies are probably directed against unique antigenic determinants localized in the central, divergent region.

**Cellular location of *T. vaginalis* alpha-actinin.** Anti-alpha-actinin antibodies were eluted from the purified recombinant plaques incubated with rabbit hyperimmune anti-*T. vaginalis* serum. Monospecific antibodies were then used to localize alpha-actinin in both pear-shaped and amoeboid, adherent forms of the parasite to investigate its participation in morphological changes. Live *T. vaginalis* cells (>99% viability) grown in suspension and bound on a solid support were fixed and examined by immunofluorescence. Figure 4 shows the immunofluorescence patterns of alpha-actinin in pear-shaped (Fig. 4A) and amoeboid (Fig. 4D) trichomonad cells and in two intermediate stages (Fig. 4B and C). Diffuse, pale fluorescence was observed in the pear-shaped parasites (Fig. 4A), suggesting that intracellular alpha-actinin was located throughout the cytoplasm. When *T. vaginalis* cells bind to a solid support and transform into an amoeboid morphology, an intracellular redistribution of alpha-actinin occurs. As shown in Fig. 4D, the protein fluorescence in amoeboid parasites was observed only in the peripheral, submembranous regions of the trichomonad cell. In intermediate stages, the protein was located at the periphery of the cell (Fig. 4B) and then appeared to be present only in cell protrusions produced during spreading (Fig. 4C). These findings strongly suggest that the protein participates in the formation of pseudopodal extensions and in transformation into the amoeboid morphology.

In order to assess whether the redistribution of *T. vaginalis* alpha-actinin is related to actin redistribution in the same cel-
lular regions, a colocalization experiment was performed. Anti–protozoan alpha-actinin and actin antibodies were coincubated with fixed amoeboid parasites adhering on coverslips. Bound anti–alpha-actinin antibodies were identified with fluorescein-conjugated antibodies, while bound antiactin antibodies were identified with rhodamine-conjugated antibodies. The two proteins were observed to colocalize in the same cellular regions of amoeboid parasites. Both actin and alpha-actin were in fact located in pseudopods and adhesion plaques (data not shown). Preimmune serum and secondary antibodies, used as controls, did not produce any fluorescence of the trichomonad cells.

Expression of trichomonad alpha-actinin mRNA. The changes in intracellular distribution observed for alpha-actinin by immunofluorescence led us to assess whether transformation into the amoeboid morphology involved only the intracellular redistribution of an already existing reservoir of alpha-actinin or whether it was accompanied by enhanced expression...
of the alpha-actinin gene. Total RNA was extracted from pear-shaped protozoa grown in suspension and from adherent amoeboid protozoa. Blots of electrophoresed RNA were probed with digoxigenin-labeled probes designed from the T. vaginalis alpha-actinin and actin nucleotide sequences. As shown in Fig. 5, this technique allowed detection of alpha-actinin transcripts only in RNA from amoeboid protozoa (Fig. 5, panel 2, lane b), while actin RNA was detected in equal amounts in both pear-shaped and amoeboid parasites (panel 1, lanes a and b). Ethidium bromide-stained duplicate gels confirmed the presence of equal amounts of total RNA in all lanes. The constant expression of the actin gene was also observed by RT-PCR (Fig. 5, panel 3, lanes a and b), while the differential expression of the alpha-actinin gene was confirmed by the same technique (panel 4, lanes a and b). RT-PCR analysis allowed us to observe that baseline transcription of the alpha-actinin gene occurs in pear-shaped protozoa and to confirm that it undergoes a dramatic increase upon transformation of the protozoan morphology.

**DISCUSSION**

Important changes in parasite morphology have been demonstrated to occur during T. vaginalis parasitism and colonization of the vaginal epithelium (5). These changes involve transformation of T. vaginalis from an ellipsoidal shape to an amoeboid morphology. The morphological transformation involves signalling and complex intracellular pathways that result in the formation of aggregates of flattened parasites bound to target cells. After transformation and adhesion, target cells are damaged and eventually lysed (16). The ability to undergo morphological changes is presumed to be related to virulence (5). In order to understand the mechanisms which mediate cytopathogenicity, it is important to investigate the cytoskeleton of the microorganism and the mechanisms that regulate the redistribution of its molecular components. That cytoskeletal integrity is important for T. vaginalis cytopathogenicity has been highlighted in the past by Juliano and coworkers (20), who showed that participation of the trichomonad cytoskeleton in interactions with target cells is required, as inferred from the effects of drugs that disrupt cytoskeletal complexes.

In protozoa, several actin-binding proteins participate in movement and morphological changes. In E. histolytica, for example, actin-binding proteins play a pivotal role in movement and cellular interactions with the environment (17, 34). It is interesting that T. vaginalis is a flagellated protozoan parasite and that its locomotion is based on flagella; its ability to transform into an amoeboid form is not necessary for movement but is required for cytopathogenicity. Therefore, studying the cytoskeletal organization of this parasite and the mecha-

![FIG. 4. Distribution of alpha-actinin in different morphological forms of T. vaginalis. Living T. vaginalis organisms in suspension and adhering on coverslips were fixed and processed for immunofluorescence staining with anti-T. vaginalis alpha-actinin antibodies. (Right panels) Immunofluorescence patterns of alpha-actinin during different morphological stages of the parasites: A, pear-shaped form; D, fully amoeboid form; B and C, intermediate stages. (Left panel) Phase-contrast micrographs of the corresponding samples. This sequence of images shows the redistribution of alpha-actinin in the periphery of the microorganism following transformation into the amoeboid morphology.](image1)

![FIG. 5. Representative experiment showing a Northern blot and RT-PCR of total mRNA extracted from pear-shaped (lanes a) and amoeboid (lanes b) T. vaginalis organisms. (Northern blot) Panel 1, hybridization bands obtained with an actin probe; panel 2, hybridization bands obtained with an alpha-actinin probe. (RT-PCR) Panel 3, amplification bands obtained from RNA extracted from pear-shaped and amoeboid parasites with actin primers; panel 4, amplification bands obtained with alpha-actinin primers.](image2)
nisms that regulate the redistribution of cytoskeletal components is of outstanding importance for understanding the mechanisms of pathogenicity.

In this work, we report the nucleotide sequence and molecular characterization of a cDNA coding for \textit{T. vaginalis} alpha-actinin. As far as we know, this is the first alpha-actinin sequence reported for a protozoan. Moreover, \textit{T. vaginalis} diverged relatively early, and detailed knowledge of its cytoskeletal organization and components represents a useful tool in terms of evolutionary studies.

The analysis performed on the sequence obtained from the cloned cDNA revealed homology with alpha-actinins of different species. Homology with other proteins belonging to the actin-binding protein family was also detected; among them, spectrin displayed the highest homology.

The central region of the protein showed less significant homology with alpha-actinins or related proteins. A less conserved central region is found among all actin-binding proteins of the spectrin family; its function is to confer a rod-like structure to this portion of the molecule and is generally the result of shuffling and duplications that occurred during evolution (29).

Analysis of the protein for antigenic determinants revealed that this region contains three high-probability regions of antigenicity. When a \textit{T. vaginalis} total protein extract was injected into rabbits, consistent production of anti-alpha-actinin antibodies was observed, demonstrating the high immunogenicity of the protein. Moreover, data from a study that we performed on patients suffering from trichomoniasis showed that patient sera displayed a strong antibody response to trichomonal alpha-actinin (1a). A test for cross-reactivity was performed on human and several protozoan protein extracts with antibodies directed against the trichomonal protein. An absence of cross-reactivity was observed; human epithelial cells and \textit{G. lamblia}, \textit{E. histolytica}, \textit{A. castellani}, and \textit{L. major} total protein extracts were not recognized by monospecific anti-\textit{T. vaginalis} alpha-actinin antibodies.

Immunofluorescence analysis demonstrated that \textit{T. vaginalis} alpha-actinin was present throughout the cytoplasm of pseudopod-shaped organisms. Interestingly, when the parasites transformed to the amoeboid morphology, high levels of alpha-actinin (1a). A test for cross-reactivity was performed on human and several protozoan protein extracts with antibodies directed against the trichomonal protein. An absence of cross-reactivity was observed; human epithelial cells and \textit{G. lamblia}, \textit{E. histolytica}, \textit{A. castellani}, and \textit{L. major} total protein extracts were not recognized by monospecific anti-\textit{T. vaginalis} alpha-actinin antibodies.

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