25

TRICHOMONAS VAGINALIS ADHESIN PROTEINS DISPLAY MOLECULAR MIMICRY TO METABOLIC ENZYMES

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1. INTRODUCTION

Trichomonas vaginalis is a flagellated protozoan responsible for trichomonosis, one of the most prevalent sexually transmitted diseases. Women are mainly affected and have a broad range of symptomatology; minor irritation to severe inflammation of the vaginal epithelium, accompanied with discharge, itching and abdominal pain have been reported (Krieger *et al.*, 1990). Because of these varied symptoms and the lack of a good clinical test, diagnosis is less than adequate with approximately 50% of women misdiagnosed.

Recent findings indicate trichomonosis presents more of a problem than previously believed. Recommended treatment for this disease is metronidazole, but drug-resistant isolates have emerged (Müller *et al.*, 1980). During the current HIV epidemic, it has been observed that patients infected with *T. vaginalis* are more likely to seroconvert to HIV positive (Nzila *et al.*, 1991; Laga *et al.*, 1993). Also, women infected with *T. vaginalis* are at increased risk for preterm rupture of membranes, preterm delivery, and low birth weight infants (Cotch *et al.*, 1991; Minkoff *et al.*, 1984; Read and Klebanoff, 1993).

T. vaginalis is a non-self-limiting infection in women, illustrating the adaptation by the parasite for survival in the adverse vaginal environment. Recently several *T. vaginalis* virulence factors have been identified. Since the vaginal environment is nutrient-limiting, it is not surprising that trichomonads have receptors that specifically bind to host proteins as part of an elaborate nutrient-acquisition system. *T. vaginalis* parasites are unable to synthesize or modify lipids (Lund and Schorb, 1962; Roitman *et* al., 1978; Lindmark, 1983; Holz *et al.*, 1987). Thus, receptor-mediated binding of apoprotein CIII of lipoproteins (Peterson and Alderete, 1984a; b), coupled with adhesin binding and subsequent hemolysis of erythrocytes (Lehker *et al.*, 1990; Dailey *et al.*, 1990) represent excellent sources of lipids. It has been shown that purified lipoproteins derived from serum or lipids extracted from erythro-

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cyte membranes can be used in lieu of serum in the complex growth medium (Lehker *et al.*, 1990).

A repertoire of low-iron-induced surface proteins have also been identified as the receptors for several iron-binding and iron-containing proteins (Lehker *et al.*, 1990). *T. vaginalis* binds lactoferrin, ferritin, cytochrome c and hemoglobin by specific receptors (Peterson and Alderete, 1984c). Among the best studied is the lactoferrin receptor (Peterson and Alderete, 1984c), and it has been shown that lactoferrin is an *in vivo* source of iron for the parasite (Masson *et al.*, 1966). Transferrin, another iron-binding protein of plasma, is not a source of iron for trichomonads (Peterson and Alderete, 1984c), illustrating the highly evolved nature for utilization of specific iron sources. The *in* vivo fluctuations in lactoferrin parallel the hormonal changes (Cohen *et al.*, 1987), and this has been suggested to represent an important *in vivo* environmental cue regulating a variety of parasite responses, such as growth rates and expression of immunogens and adhesins (Lehker *et al.*, 1991; Lehker and Alderete, 1992).

Up to twenty-three cysteine proteinase activities have been identified by two-dimensional substrate electrophoresis (Neale and Alderete, 1990). That all proteinases are expressed *in vivo* is exemplified by the detection of serum antibodies to most, if not all, cysteine proteinases in patients with trichomonosis (Alderete *et al.*, 1991a; b). Interestingly, evaluation of proteinase patterns of freshly isolated parasites showed only subsets of the entire repertoire (Neale and Alderete, 1990; Alderete *et al.*, 1991b), perhaps indicating environmental control of the expression of these genes. The proteinases, as well as anti-proteinase antibodies have been detected in vaginal washes of patients (Alderete *et al.*, 1991b).

The significance of so many cysteine proteinases within trichomonads may ultimately be determined through substrate specificity studies. Recent work has shown that some are involved in the degradation of immunoglobulins (Provenzano and Alderete, 1995). An iron-regulated proteinase has also been found to render otherwise highly susceptible trichomonads resistant to lysis by the C3 component of complement (Alderete *et al.*, 1995b). The ability to degrade these proteins is likely protective for trichomonads, and also has implications for immunity/vaccines against co-infecting STDs. Hemolysis and contact-dependent cytolysis have been shown to involve trichomonad cysteine proteinases (Dailey *et al.*, 1990; Arroyo and Alderete, 1995). The expression of substrate-specific proteinases, as mentioned above, is under the control of environmental cues at the site of infection (Alderete *et al.*, 1995b; Provenzano and Alderete, 1995).

Among ethnic populations, African Americans have higher rates of trichomonosis (Stevens-Simon *et al.*, 1994). Approximately 50% of *T. vaginalis* isolates contain a segmented double-stranded RNA virus (Khoshnan and Alderete, 1993); recent unpublished observations suggest a relationship between virus-harboring *T. vaginalis* isolates and infection of African American women. This may be of particular importance, since African American women appear at risk for adverse outcomes in pregnancy and low-birth-weight infants (Bramley, 1976).

Only isolates with the virus are capable of the interesting phenomenon of phenotypic variation, as shown for a highly immunogenic protein called P270 (Wang *et al.*, 1987; Alderete *et al*, 1986a; b). These isolates vary between surface and cytoplasmic expression of P270. Virus-minus trichomonal isolates synthesize P270, but are unable to surface express the immunogen. That this variation represents an immune evasion technique is evidenced by the fact that antibody in patient serum to P270 is parasiticidal (Alderete and Kasmala, 1986). Finally, a recent report documented viral up-regulation of P270 (Khoshnan and Alderete, 1994), and regulation by the virus of other trichomonad proteins also has been demonstrated (Provenzano and Alderete, unpublished results).

As mentioned above, cytoadherence is one of the early steps essential for colonization and persistence of *T. vaginalis* in the human urogenital tract. All mucosal pathogens must

overcome host factors and responses in order to colonize the mucosa and establish infection. Some of these factors include the extensive mucus layer, nutrient-limiting conditions, immune surveillance, and the constant fluid flow of the vagina. The complexity of this host environment is further illustrated by the hormonal influences at the site of infection.

T. vaginalis has evolved adaptive responses, such as adherence to host cells, which aid in the establishment of infection. Overall, the emerging picture of trichomonal cytoadherence suggests a very complex series of steps, and in our opinion, this property is more accurately viewed as a cascade set of reactions rather than a simple ligand-receptor interaction. For instance, one or more cysteine proteinases is required for parasite cytoadherence (Arroyo and Alderete, 1989; 1995). It has also been shown that iron regulates adhesin synthesis at the transcriptional level (Lehker *et al.*, 1991). Furthermore, binding to host cells results in distinct signals received by the parasite (Arroyo *et al.*, 1993). First, motile trichomonads, upon contact with vaginal epithelial cells, undergo a morphologic transformation and convert to an ameboid form, possibly for enhanced association with host cell surfaces. Second, trichomonad adhesin synthesis is rapidly up-regulated following contact with the host cell. Third, other trichomonads are recruited to the site of contact on the vaginal epithelial cell. These observations reinforce the concept that *T. vaginalis* attachment is a complex process and shows the ability of the parasite to be responsive to the constantly changing vaginal environment.

It has been shown previously that *T. vaginalis* adherence to host cells is a highly specific event that indicates parasite-cell ligand-receptor type associations (Alderete and Garza, 1985; 1987; Alderete *et al.*, 1988). We established that cytoadherence is time, temperature, and pH dependent (Alderete and Garza, 1985; 1987). Also, *T. vaginalis* organisms demonstrated a tropism for epithelial cells (Alderete and Garza, 1985). Loss of the ability to cytoadhere after live organisms were treated with trypsin, and prevention of cytoadherence regeneration by inhibition of protein synthesis indicated that parasite surface proteins were involved in host cell recognition (Alderete and Garza, 1985).

The *T. vaginalis* adhesins were finally identified through the use of a ligand assay (Alderete and Garza, 1988; Lehker *et al.*, 1991; Arroyo *et al.*, 1992) in which a detergent extract of intrinsically or extrinsically-radiolabeled parasites was incubated with chemically-stabilized host cells. Avidly bound proteins were eluted and resolved on SDS-PAGE-fluorography or autoradiography. Four trichomonad proteins capable of binding to host cells were identified and designated AP65, AP51, AP33 and AP23 based on M_r (Fig. 1A). Various established criteria (Beachey, 1989), some of which are summarized in Table I, were satisfied to verify that these proteins are bona fide adhesins (Arroyo *et al.*, 1992).

Polyclonal antiserum and monoclonal antibody (mAb) were generated against each of these proteins (Fig. 1A). Screening of a phagemid expression library with these antibodies yielded several reactive clones; three for AP65, one for AP51 and six for AP33 (Arroyo *et al.*, 1995). The expression patterns of the recombinant proteins in *E. coli* are shown in figure 1B. The readily visible bands for the AP51 and AP33 clones, in comparison to control *E. coli* proteins, verify the overexpression of these recombinant proteins. In contrast, the recombinant AP65 proteins were not apparent by Coomassie brilliant blue staining, but could be detected by immunoblotting.

The functionality of each of the recombinant proteins was then readily established. The immunoblot in figure 2A shows that a representative of each of the recombinant proteins was capable of binding to host cells, as did the natural parasite adhesin. Importantly, as shown in figure 2B, the representative recombinant proteins inhibited binding of the respective native trichomonad adhesins (Arroyo *et al.*, 1995).

Cloning the genes encoding the adhesins, and expression of functional recombinant proteins are essential first steps toward understanding interrelationships between the adhesins, as well as the nature of the interactions with host cell receptors. The initial charac-

Table 1. Four *T. vaginalis* surface proteins function as adhesins

1. Four distinct, non-immuno-crossreactive surface proteins of *T. vaginalis* bind to host cells.
2. A direct relationship between amounts of the four surface-expressed proteins and cytoadherence levels was established.
3. The four purified proteins and antibody to each of the four proteins inhibits cytoadherence in a concentration-dependent fashion.
4. Host cells have a saturable number of receptors for the four proteins.
5. Recombinant proteins retain function and inhibit binding of the natural trichomonad proteins to the host cell surface.

terization of genes encoding three of the *T. vaginalis* adhesins is presented here. Each of these adhesins was found to be encoded by a family of genes that share homology with metabolic enzymes; evidence is presented strongly suggesting that expression and subsequent protein localization of the adhesins may be regulated by multiple mechanisms. This data illustrates an added level of complexity for these important biofunctional proteins.

2. MOLECULAR CHARACTERIZATION OF THE AP65 GENES

2.1. Multiple-Gene Family

2.1.1. The cDNAs Represent Three Unique Genes. Insert analysis revealed that two of the clones were of sufficient size to encode the full-length adhesin, while the third was truncated and missing approximately one-third of the gene. Surprisingly, restriction enzyme mapping showed that, although similar, each of the cDNAs had clear differences. Subsequent

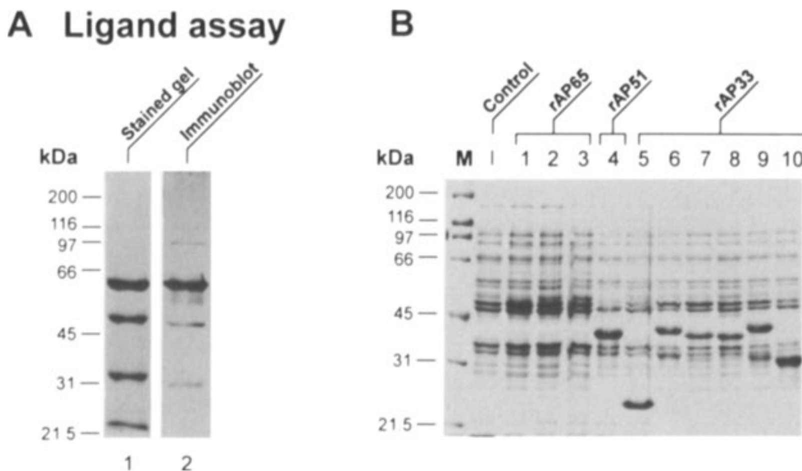

Figure 1. Analysis of trichomonad adhesins (A) and the recombinant proteins expressed in *E. coli* (B). (A) Trichomonad adhesins detected in stained gels after the ligand assay (lane 1) or after immunoblotting of adhesins with a mixture of antisera to the four adhesins. (B) Commassie brilliant blue-stained total proteins of control *E. coli* lysates (lane labeled control) were compared with proteins in lysates of recombinant *E. coli* (lanes 1 through 10). Intense recombinant protein bands specific for cDNA clones 4 through 10 had M_rs from ~23- to 42-kDa and were not seen in control *E. coli* lysates. Molecular weight markers (BioRad) are on the left in kilodaltons (kDa) (x1000). Printed with permission of Archives of Medical Research.

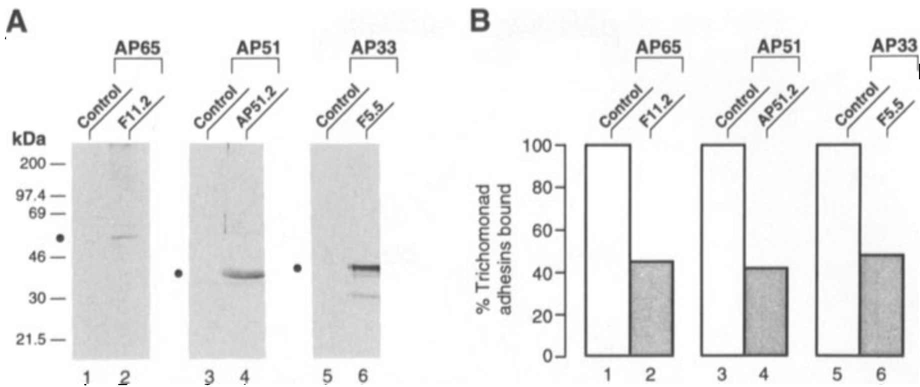

Figure 2. Representative experiments showing that recombinant proteins bind to HeLa cells (A) and compete with ^{35}S-labeled adhesins in *T. vaginalis* extract for HeLa cell binding (B). The recombinant *E. coli* lysate used for the experiments is designated above each lane. Control refers to *E. coli* harboring the vector without inserts used as negative control (lanes 1, 3 and 5). (A) Each recombinant protein bound and released from HeLa cell surfaces was electrophoresed and blotted onto nitrocellulose for detection with specific antiserum or mAb as for Figure 1. Control prebleed rabbit serum or myeloma culture supernatant failed to detect any of the bound recombinant proteins as for Figure 1. Dots designate the major recombinant adhesin bound to fixed HeLa cells. The numbers on the left show the size in kilodaltons (kDa) (x1000) of the prestained Rainbow molecular weight markers (Amersham Corp., Arlington, IL). (B) Fixed HeLa cells were interacted first with recombinant *E. coli* lysates and then with solubilized radiolabeled trichomonads in a ligand assay. Densitometric scanning analysis of the resulting fluorogram was used to determine the percentage of ^{35}S-labeled trichomonad adhesins bound to HeLa cells. The percentage of trichomonad adhesins binding after first interacting HeLa cells with control *E. coli* lysates represents 100% binding (lanes 1, 3 and 5). This experiment was repeated three times, and representative results are shown. Printed with permission of Archives of Medical Research.

sequencing of these cDNAs confirmed that each represented a unique gene, providing insight into the existence of a multigene family for this adhesin (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

2.1.2. Obtaining and Comparing the Full-Length Sequences. Further analysis of the nucleotide sequences showed that the two largest cDNAs contained open reading frames (ORFs) that encoded for proteins of 543 amino acids, with predicted molecular masses of approximately 60,000 daltons (60-kDa). As the apparent size of the adhesin (Fig 1A) was not consistent with that predicted from the sequences, and all three cDNAs had a 3′ poly-A tail, this indicated that a portion at the 5′ end was missing from each. Since the cDNAs were similar but not identical, it was possible to identify unique oligonucleotide regions for the 5′ ampliFINDER RACE method to obtain the 5′ ends. Sequence analysis revealed a new translational start site, with each of the ORFs being identical in size. The complete sequences coded for proteins of 567 amino acids with predicted molecular masses of 63.3-kDa, 63.1-kDa, and 63.1-kDa, consistent with the native AP65 adhesin. Comparisons between the sequences showed 87%, 88% and 96% identity at the nucleotide level, and 91%, 89% and 96% identity for the proteins (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

2.1.3. Each Gene Is Multi-Copy. The 5′-end PCR products described above and the highly specific oligonucleotide used to generate the 5′-end sequence for the truncated, third cDNA were shown to hybridize only with the corresponding cDNA from which it was derived. This gave us gene-specific probes for Southern analysis of genomic DNA of individual isolates, as well as of agar clones derived from single trichomonads. The DNA was restricted with enzymes that do not cut within the AP65 genes. Unexpectedly, each of the probes generated multiple bands, indicating that the three genes are present in the *T. vaginalis* genome and likely in multiple copies (Arroyo *et al.*, 1995; Alderete *et al.*, 1995a; O'Brien *et al.*, 1996). The data show that the AP65 adhesin is a member of a multigene family, each of which is present in the genome of all trichomonads.

2.1.4. Each of the Recombinant AP65s has Properties Similar to the Native Adhesin. Each of the recombinant proteins was immunoreactive with polyclonal antibodies and mAbs to the natural AP65 obtained from the ligand assay. Furthermore, each recombinant AP65 was tested for functionality and found to be capable of binding to host cells. The binding of native AP65 was inhibited by each recombinant protein, indicating that each of the cDNAs encoded a functional, yet distinct adhesin protein (Arroyo *et al.*, 1995; Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

2.2. AP65 Has Homology to Malic Enzyme

2.2.1. Database Search Reveals Homology to Malic Enzyme. Database analysis found significant similarity between the AP65 clones and the decarboxylating malic enzymes. Malic enzyme is active in metabolic pathways and present in the trichomonad hydrogenosome, the anaerobic equivalent of mitochondria (Lahti *et al.*, 1992). All AP65s showed ~54% identity at the nucleotide level and 38% identity at the amino acid level when compared to human malic enzyme. However, there were four regions in which the AP65s were almost identical, and also showed a high degree of identity, up to 78%, with malic enzyme. It may be important that the amino acid positions of all these regions are similar for the AP65 clones and the malic enzymes (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

2.2.2. The Malic Enzyme Conserved Domains. Three of the four conserved regions correspond to dinucleotide-binding sites identified in several enzymes, and the other is a divalent cation-binding site of malic enzyme. The first region is the putative NADP-binding site of human and murine malic enzyme, goose fatty-acid synthetase, and human glyceraldehyde 3-phosphate dehydrogenase (Loeber *et al.*, 1991; Bagchi *et al.*, 1987; Poulouse and Kolattukudy, 1983). The AP65s show homology to 7 of the 9 malic enzyme amino acids spanning this domain. A cysteine residue believed to be the malate-binding site (Satterlee and Hsu, 1991) immediately follows this domain, and is conserved among all malic enzymes, as well as the AP65s. It is likely that this cysteine is important in binding of L-malate, but not NADPH, since modification of the residue only affects malate binding (Satterlee and Hsu, 1991).

The second conserved domain is the ADP-binding βαβ fold (Wierenga *et al.*, 1985). A consensus sequence (Wierenga *et al.*, 1985) consisting of hydrophilic and hydrophobic residues, as well as a highly conserved six amino acid motif has been established from sequence analysis of various enzymes, including human and duck malic enzymes, horse alcohol dehydrogenase, dogfish lactate dehydrogenase, and lobster glyceraldehyde phosphate dehydrogenase (Loeber *et al.* 1991; Hsu *et al.*, 1992; Wierenga *et al.*, 1985). This motif, GXGXXG, is essential for NAD-binding (Scrutton *et al.*, 1990). The AP65s follow the motif

exactly, and also show a high degree of homology to the rest of the consensus sequence (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

The third conserved dinucleotide-binding fold includes the GXGXXA sequence necessary for the binding of NADP in human and duck malic enzyme, human adrenodoxin reductase, *Agrobacterium* octopine synthase, and *E. coli* glutathione reductase (Loeber *et al.*, 1991; Hsu *et al.*, 1992; Scrutton *et al.*, 1990). The AP65s all conform perfectly to this motif as well as following the general pattern surrounding it (Alderete *et al.*, 1995a; O'Brien *et al*,.,1996).

Finally, alignment of the AP65s corresponded well with the putative Mn^{2+}-binding site of malic enzymes (Wei *et al.*, 1994), including pigeon, human, duck, murine, *Ascaris suum*, and maize (Wei *et al.*, 1994; Loeber *et al.*, 1991; Hsu *et al.*, 1992; Bagchi *et al.*, 1987; Kulkarni *et al.*, 1993; and Rothermel and Nelson, 1989). This domain includes a highly conserved aspartic acid believed to be one of the Mn^{2+} ligand sites of malic enzyme.

2.2.3. Malic Enzyme Does Not Exhibit Adhesive Function. It was of interest to determine if malic enzyme had properties similar to the AP65 adhesins. A monoclonal antibody generated against AP65 was found to be immuno-crossreactive with commercially-purchased malic enzyme, indicating recognition of a common epitope. However, malic enzyme was not able to inhibit binding of native AP65 or the recombinant AP65s to host cells in a ligand assay, even at high concentrations. Likewise, malic enzyme did not compete with live trichomonads for host cell binding sites in a cytoadherence competition experiment, suggesting that the four conserved domains are insufficient for adherence to host cells (Alderete *et al.*, 1995a).

2.3. Regulation of Expression/Localization

2.3.1. Iron Regulation. Earlier work had shown that iron regulated cytoadherence and synthesis of the *T. vaginalis* adhesins (Lehker *et al.*, 1991). The isolation of three different and independent clones indicated *a priori* that each of the three AP65 genes was transcribed. Northern analysis of RNA isolated from trichomonads showed that the specific probes hybridized to transcripts of ~1.8 kb, a size consistent with the AP65 genes. Interestingly, transcripts were only detected in total RNA from high-iron-grown trichomonads. Detection of transcripts in RNA derived from low-iron-grown organisms required prolonged exposure of X-ray film, indicating that trichomonads maintain basal levels of each transcript. Therefore, iron plays a role in upregulating transcriptional expression of each AP65 gene. Transcripts of all three genes were seen in the parental population as well as all clones, confirming the presence of each gene in all trichomonads, and suggesting that each gene is expressed within individual trichomonads (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996).

2.3.2. Promoter Regions. The 5′ untranslated regions of the AP65 clones were fairly short at 13 to 17 nucleotides, and did not contain any of the typical eukaryotic promoter elements, such as the TATA box. Sequence analysis 5′ to the start site showed similarity to the promoter elements of other reported *T. vaginalis* protein-coding genes, including ferredoxin, β succinyl-CoA synthetase, α succinyl-CoA synthetase, α-tubulin, β-tubulin, 70-kDa heat-shock protein, and P-glycoprotein 1 (Quon *et al.*, 1994). Based on these genes, a 13 bp consensus sequence for *T. vaginalis* promoters, TCAYTWYTCATTA, has been defined (Quon *et al.*, 1994). Alignment of the AP65s with this sequence showed that between 8-10 bases of each AP65 5′ end conformed to the pattern. Of particular interest was the observation that each of the AP65 promoters have differences at the -9 and -10 positions (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996). These gene-specific bases may affect RNA polymerase

recognition of the promoters, and therefore play a role in transcriptional regulation of the AP65 adhesins.

2.3.3. 3′ Untranslated Regions (UTRs). The AP65 genes show little or no homology in the 3′-UTRs, the sequence from the stop codon to the poly-A tail. It is interesting that two of the transcripts for the AP65 genes have similar features putatively involved in destabilizing mRNA. Both have short 15 bp poly-A tails, easily degraded relative to the third transcript, which has a 55 nucleotide poly-A tail. Both also contain the sequence motif ATTTA in the cDNA, or AUUUA in mRNA, found in AU-rich elements (AREs); these elements have been shown to confer instability on mRNAs (McCarthy and Kollmuss, 1995). A separate report suggested that additional surrounding bases were also important for destabilization, lengthening the sequence to UUAUUUAU (Zubiaga *et al.*, 1995). This more complete destabilizing sequence was found in the 3′-UTR of one of the AP65 genes (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996). Therefore, it is possible that each of the AP65 transcripts are subject to translational regulation through differential degradation of select AP65 transcripts.

2.3.4. Leader Sequences. From N-terminal amino acid sequencing it was determined that the mature AP65 polypeptides were missing twelve amino acids at the 5′ ends, thus identifying potential signal sequences (Alderete *et al.*, 1995a). A comparison of the signal peptides to other known leaders revealed similarities to *T. vaginalis* hydrogenosomal protein signal sequences and mitochondrial leaders (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996; Lahti *et al.*, 1992; von Heijne *et al.*, 1989). Consistent with previous reports, all of the sequences begin with Met-Leu and have an arginine at the -2 position relative to the cleavage site (Lahti *et al.*, 1992). It is possible that the differences in the signal peptides, albeit slight, selectively localize each of the AP65s to distinct cellular sites.

3. INITIAL CHARACTERIZATION OF AP51 AND AP33

This section is based on preliminary, unpublished data.

3.1. Multi-Gene Families

3.1.1. Three Similar Yet Unique Genes Exist for AP51 and AP33. Restriction enzyme analysis and sequence data confirmed that the six clones for AP33 represent 3 similar, yet distinct, genes. The clones contain inserts that encode near full-length proteins for each, and procedures similar to those used for AP65, described above were used to get complete gene sequences. Homology between these AP33 genes is 91.8%, 92.7% and 95.9%, and between the proteins is 97%, 97.3% and 99.3% respectively, indicating the highly conserved nature of this adhesin.

Only one antibody-reactive cDNA clone was isolated for AP51, and insert analysis, along with sequence information indicated approximately one-third of the 5′ end was missing. While trying to generate the 5′ end for this gene, evidence was obtained for the existence of two additional AP51 genes. This data demonstrates that the three *T. vaginalis* adhesin proteins so far studied are encoded by multigene gene families and, importantly, each family exists within individual trichomonads.

3.1.2. The Genes Encode Functional Adhesin Proteins. We had already shown that the AP51 clone and one of the AP33 clones bound to host cells and efficiently inhibited the

corresponding native adhesin from binding (Arroyo *et al.*, 1995). The two additional AP33 proteins were tested for these activities and were found to behave similarly.

3.2. Homology to Succinyl-CoA Synthetase. Database searches of reported DNA and protein sequences revealed that the AP33 genes and the AP51 gene have homology to the α and β subunits of succinyl-CoA synthetase, respectively. As with AP65, these homologous proteins are believed to be active in a metabolic pathway and have also been found in the *T. vaginalis* hydrogenosome (Lahti *et al.*, 1992; Lahti *et al.*, 1994). Proteins encoded by the three AP33 genes have 53.8%, 54.5%, and 54.9% identity with the α subunit of *E. coli* succinyl-CoA synthetase (Buck *et al.*, 1985). The AP51 clone shows 44.2% identity to the β subunit at the amino acid level (Buck *et al.*, 1985). There are three conserved domains in the α subunit which may contribute to nucleotide binding. Each AP33 gene is highly homologous to the enzyme subunit in these domains. There is also a conserved region surrounding a histidine residue, which, when phosphorylated, gives rise to the active form of the enzyme (Lou and Nishimura, 1991; Majumdar *et al.*, 1991), and this residue is present in the AP33 proteins.

3.3. Regulation of Expression/Localization

3.3.1. Iron Regulation. Although unique probes for each of the AP33 and AP51 genes have not yet been generated, Northern analysis revealed that transcripts for AP33 and AP51 were only detected in RNA isolated from parasites grown in iron-replete medium (Arroyo, *et al.*, 1995). This suggests that, as for AP65, transcription of each of these genes is regulated by iron.

3.3.2. Promoter Sequences. Although each AP33 promoter is unique, the sequences follow that reported for other *T. vaginalis* genes (Quon *et al.*, 1994). To date, the promoter for only one of the AP51 genes has been identified, and interestingly, it is identical to one of the AP33 promoters, but different from the AP65 promoters. It is not unlikely that the subunits of an enzyme would have the same promoter element, however, it is unknown at this point whether AP33 and AP51 form a complex during the adherence event. Each protein/subunit is capable of adhering to host cells and inhibiting native adhesin binding, suggesting that such a complex is not necessary for adhesin activity. However, using the same promoter may ensure generation of equal amounts of transcripts for at least one of the AP33 and AP51 proteins.

3.3.3. 3′ UTRs. Major differences between each of the AP33 3′ UTRs confirms the presence of three distinct genes. This is also the case for the three AP51 genes, confirming that both of these adhesins are encoded by multigene families. Although none of the AP33 genes contains the destabilizing AREs, one of the AP51 genes does. One gene for AP33 and one for AP51 have the same promoter element, and this implies that these genes are coordinately regulated at the transcriptional level. This raises the possibility that these genes may also be coordinately regulated at the translational level. Therefore, more genes may exist for either AP33, or both AP51 and AP33 if the proteins function as subunits in a complex and share similar regulatory mechanisms.

3.3.4. Leader Sequences. As for the recombinant AP65s, leader sequences were identified for each of the AP33 proteins, and these peptides were similar to other known

leader sequences (Alderete *et al.*, 1995a; O'Brien *et al.*, 1996; Lahti *et al.*, 1992; von Heijne *et al.*, 1989). Two of these signal peptides were identical, indicating a possibility for similar localization of two AP33 proteins within the organism, while the third may be trafficked to other areas. The 5′ ends of the AP51 genes, including sequences encoding potential leader peptides, are still being generated.

4. CONCLUSIONS AND QUESTIONS

This overview of ongoing research activities summarizes the molecular characterization of *T. vaginalis* proteins involved in adherence to host cells. Results show that multiple-gene families encode three of four trichomonad adhesins, referred to as AP65, AP51, and AP33. That we have discovered three members, or isoforms, of each multigene family and that each of those genes is present in multiple copies reaffirms the importance and significance of these proteins to the overall biology of this parasite in general, and the property of cytoadherence in particular.

Iron is an *in vivo* signal to *T. vaginalis* (Lehker and Alderete, 1991; 1992), and iron-limited organisms provided with iron increase transcription and synthesis of adhesins (Lehker and Alderete, 1991). Adherence to host cells represents another signal; immediately after contact, the parasites undergo a dramatic morphological transformation concomitant with the rapid synthesis of adhesins (Arroyo *et al.*, 1993). That each of the adhesins is encoded by more than one gene may insure for the expression and synthesis of adequate amounts of adhesins within a short period of time following contact. Also possible is that the different copies of each adhesin gene, although coordinately regulated by iron, respond to other yet undefined environmental signals. It is conceivable, for example, that only one or two of the genes for each adhesin are directly involved in transcription and expression of the adhesin following contact with the host cell surface (Arroyo *et al.*, 1993). The other gene(s) may be responsive at times other than contact, for instance when amounts and types of vaginal iron sources fluctuate, such as during menstruation. Thus, it is essential that the arrangement and regulation of each member of the adhesin gene family be examined further.

Unexpectedly, each of the three *T. vaginalis* adhesins show significant homology to metabolic enzymes known to exist in the trichomonad hydrogenosome. It has not yet been determined whether the trichomonad adhesins express enzymatic activity. Nevertheless, in inhibition experiments using commercially available malic enzyme as an analog for AP65, the enzyme did not interfere with *T. vaginalis* cytoadherence to host cells, showing that the receptor-binding epitope is a sequence unique from the regions conserved among malic enzymes (Alderete *et al.*, 1995a).

Although percent identities between the gene members of each adhesin family are high, as much as 99% identity between the predicted amino acid sequences of two AP33 genes, the resulting variation in amino acids may result in differences in function. In the case of the *fimH* gene of *Escherichia coli*, gene variants displaying more than 98% homology and encoding proteins that differ by as little as one amino acid confer distinct adhesive phenotypes (Sokurenko *et al.*, 1994). Thus, the minor sequence variations among members of a *T. vaginalis* adhesin gene family may confer enzymatic activity for one protein and adhesive capacity for another. Alternatively, the divergence in sequence may lead to differences in receptor specificity. Functional assays of the recombinant proteins will clarify the effects of sequence variations.

We would be remiss to exclude the possibility that one of the members of each adhesin family represents the corresponding trichomonad enzyme. Comparison of the adhesin gene sequences with the reported enzyme sequences reveals that a number of the genes are

Table 2. Enzymes on mammalian cells and functions outside the metabolic pathways

α-enolase	plasminogen receptor
Lens proteins/crystallins	structural lens proteins
1. lactate dehydrogenase 2. alcohol dehydrogenase 3. hydroxyacyl CoA dehydrogenase 4. NADPH-dependent reductases 5. α-enolase 6. glutathione S-transferase 7. argininosuccinate lyase	

References cited in text.

identical. Recent reports present the sequences of *T. vaginalis* malic enzyme (Hrdy and Müller, 1995), and the α (Lahti *et al.*, 1994) and β (Lahti *et al.*, 1992) subunits of succinyl-CoA synthetase. However, transcripts from these genomic copies have not been shown to yield proteins with enzymatic activity. It is critical to determine whether the hydrogenosomal enzymes and the trichomonad adhesins are, in fact, encoded by identical genes, a phenomenon known as gene sharing (Piatigorsky and Wistow, 1989). The adhesin proteins may possess bifunctionality, having both the catalytic function of metabolic enzymes and adhesive properties. Although this very well may be the first report of a protozoan using surface-expressed metabolic enzymes as adhesins, this phenomenon is not without precedence among other microbial pathogens, as detailed below.

Some of the earliest reports of enzymes having important roles beyond those in metabolism (Table 2) were the crystallins, the structural proteins of the lens, which were revealed to be metabolic enzymes (Piatigorsky and Wistow, 1989; Wistow, 1993; Wistow and Piatigorsky, 1987). One of the crystallins, α-enolase, has also been implicated as a plasminogen receptor when surface-expressed (Miles *et al.*, 1991). A number of reports have described metabolic enzymes on the surfaces of microbial pathogens, as listed in Table 3 (Camara *et al.*, 1994; Joe *et al.*, 1994; Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992; Vacca-Smith *et al.*, 1994; Goudot-Crozel, 1989). These surface enzymes possessed multiple functions, including adherence (Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992; 1993; Vacca-Smith *et al.*, 1994). Interestingly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found on the surface of Group A Streptococci (Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992) and on the outer membrane of *Schistosoma mansoni* (Goudot-Crozel *et al.*, 1989). On Group A Streptococci GAPDH functions as a plasmin receptor (Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992), however, a number of additional functions have been described including uracil DNA glycosylase activity (Meyer-Siegler *et al.*, 1991), protein kinase activity (Kawamoto and Caswell, 1986), bundling of microtubules

Table 3. Enzymes on microbial pathogens and parasite surfaces

Streptococcus pneumoniae	neuraminidase
Porphyromonas gingivalis	glutamate dehydrogenase
Group A Streptococci	glyceraldehyde-3-phosphate dehydrogenase
Streptococcus gordonii	glucosyltransferase
Schistosoma mansoni	glyceraldehyde-3-phosphate dehydrogenase

References cited in text.

(Huitorel and Pantaloni, 1985), and binding of fibronectin, lysozyme, and cytoskeletal proteins (Pancholi and Fischetti, 1992). Another surface-localized enzyme, glucosyltransferase, has been demonstrated to mediate the adhesion of *Streptococcus gordonii* to human endothelial cells (Vacca-Smith *et al.*, 1994).

It is equally plausible that a new structural role was developed following gene duplication and subsequent separation of function (Piatogorsky and Wistow, 1989; Wistow, 1993). Moreover, features acquired after gene duplication may contribute to the evolution of the distinct protein functions. For example, since function might be determined by location, different signal peptides may direct the proteins to distinct cellular sites. We cannot exclude the possibility that the slight differences in the leader sequences selectively localize each molecule to distinct cellular locations. Alternatively, a recent report demonstrates that mRNAs contain site-directing determinants (zip-codes) for translation and expression of proteins in specific regions of the cell, a phenomenon documented for the transcripts that encode actin, tubulin and vimentin (Kislauskis and Singer, 1992). Like the AP65, AP51, and AP33 adhesins, actin is a family of almost identical proteins that differ in the 3′-UTRs. It has been proposed that the mRNAs for the different actin isoforms dictate different compartmentalization for the synthesis of each isoform (Kislauskis and Singer, 1992). The use of antibodies specific for the recombinant proteins of each adhesin in immunogold labeling of *T. vaginalis* could identify the cellular location(s) of the adhesins.

We have shown numerous times that the adhesin genes are regulated by iron (O'Brien *et al.*, 1996; Alderete *et al.*, 1995a; Lehker *et al.*, 1991; Lehker and Alderete, 1992). If, in fact, any of the adhesin proteins do represent the corresponding *T. vaginalis* enzymes, these findings show that these and possibly other hydrogenosomal enzymes are up-regulated by iron. To our knowledge, this would be the first time that the expression of trichomonad hydrogenosomal enzymes has been shown to be regulated by iron. A recent report demonstrates that changes in culture conditions result in changes in the activities of *T. vaginalis* metabolic enzymes (Ter Kuile, 1994). Thus, it is critical to consider certain factors when studying the regulation of *T. vaginalis* enzymes and, therefore, of detection of trichomonad enzymatic pathways.

Upon contact with VECs or HeLa cells, trichomonads synthesize greater amounts of all four adhesins (Arroyo *et al.*, 1993); we hypothesized that the increase in adhesin synthesis was due to utilization of internal iron pools (Arroyo *et al.*, 1993). Although speculative, it may be important to consider that adhesin synthesis is regulated by the rate of transcript turnover. Recent reports, for example, demonstrate that mRNA turnover involves specific sequences and regions that contribute to the instability of transcripts (Peltz and Jacobson, 1992; Zubiaga *et al.*, 1995; McCarthy and Kollmus, 1995; Chen and Shyu, 1994). It may be significant that the AU-rich element (ARE), a sequence element involved in RNA destabilization (Chen and Shyu, 1994; Zubiaga *et al.*, 1995), is present in the 3′-UTRs of some of the adhesin genes. The presence of these destabilizing elements in select adhesin mRNAs may suggest differential degradation of the transcripts. The signalling event described earlier may lead to a decrease in mRNA turnover, especially of the transcripts containing AREs, providing for a mechanism of rapid expression of the individual adhesin proteins.

Another possible device for the expeditious expression of all four adhesins following signalling is the packaging of the proteins into vacuoles. Considering that the four *T. vaginalis* adhesins are coordinately expressed, one might envision that all four proteins are placed within a vesicle and exported in this fashion. This would allow for rapid simultaneous surface localization of the four adhesins. Although the precise interrelationship between the adhesins remains unknown, some form of packaging within organelles for subsequent coordinated expression would be consistent with the fact that each adhesin is essential for

cytoadherence (Alderete and Garza, 1985; 1988; Arroyo *et al.*, 1992). The exact interrelationship of the adhesins once they reach the parasite surface also remains a mystery. While the four adhesins may exist on the surface separately, it is equally plausible that, particularly among the three found to have homology to enzymes, the proteins act as subunits forming adhesive complexes.

In view of the highly conserved nature of metabolic enzymes and the metabolic enzyme sequences in the adhesins, the placement of host-like proteins on the surface of this sexually transmitted pathogen may be significant and play a role in immune evasion. This would exemplify a type of molecular mimicry, something that has received increasing attention as an important mechanism by which parasites escape recognition by the host immune system (Damian, 1989). We have already reported that the adhesins are immunorecessive in nature, and preliminary results suggest that adhesin antibody, if present at all, is at low levels in vaginal wash or human serum (Alderete *et al.*, 1991b; unpublished observations). This tactic used by the parasite raises important concerns for vaccine development using biofunctional trichomonad molecules, such as the adhesins, which mimic host proteins. Localization of the receptor-binding epitope and the extent of similarity of this epitope with host sequences will be crucial in the consideration of these proteins as vaccine candidates and development of diagnostics.

The results to date prompt the following questions:

1. What is the interrelationship between the adhesins? How are they arranged on the parasite surface?
2. Are the receptors on the host-cell surface the same for all four adhesins, unique for each adhesin, or even unique for each isoform within each adhesin family?
3. Does the increase in adhesin expression following attachment to host cells result from the utilization of internal iron pools, or are there other yet undefined environmental signals? Does the signalling event lead to stabilization of otherwise degradable adhesin transcripts and hence lead to increased adhesin synthesis?
4. Are all isoforms of an adhesin family surface-expressed? If not, what signals regulate the differential expression of these proteins?
5. Which of the adhesin genes discussed here encode genuine hydrogenosomal enzymes?

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