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Cloning and molecular characterization of two genes encoding adhesion proteins involved in *Trichomonas vaginalis* cytoadherence

J. F. Alderete,[1]* Jennifer L. O'Brien,[1] Rossana Arroyo,[2] Jean A. Engbring,[1] Oxana Musatovova,[3] Omero Lopez,[1] Crystal Lauriano[1] and John Nguyen[1]

[1]*Department of Microbiology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7758, USA.*

[2]*Departamento de Patología Experimental, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, DF 07300, México.*

[3]*Institute of Radioecology and Applied Nuclear Techniques, Kosice, Slovakia.*

Summary

Cytoadherence to the vaginal epithelium is a critical step in infection by the eukaryotic flagellate *Trichomonas vaginalis*. Four trichomonad surface proteins (AP65, AP51, AP33 and AP23) mediate cytoadherence. The cDNA encoding the AP65 adhesin was isolated from a phagemid cDNA expression library by screening with antiserum and monoclonal antibody (mAb) raised against the purified trichomonad AP65 protein. Two clones, F11.2 and F11.5, coded for immuno-crossreactive recombinant proteins that possessed functional properties equal to the *T. vaginalis* AP65 adhesin. Analysis of full-length sequences corresponding to the F11.2 and F11.5 cDNAs revealed that both contained 1701-base open reading frames (ORFs) that encoded proteins of 63 281 daltons and 63 087 daltons, respectively. Comparison of the full-length sequences showed 87% identity at the nucleotide level and 91% identity at the protein level. Restriction-enzyme mapping and Southern analysis reaffirmed the distinctness of the F11.2 and F11.5 cDNAs, indicating that two different AP65 genes (now called *ap65-1* and *ap65-2*) are present in the *T. vaginalis* genome in at least two copies each. Northern analysis detected high levels of transcript of ~1.8 kb for both *ap65-1* and *ap65-2* genes in trichomonads grown only in high-iron medium, confirming the transcriptional regulation of adhesin synthesis by iron. Homology searches revealed significant similarity (38% amino acid identity and 54% nucleotide identity) to malic enzymes. However, purified malic enzyme and mAb to AP65 crossreactive with malic enzyme neither inhibited cytoadherence of *T. vaginalis* to host cells nor prevented binding of the trichomonad AP65 to HeLa cells in a ligand assay.

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Introduction

The flagellated eukaryote *Trichomonas vaginalis* is the aetiological agent of one of the most common sexually transmitted diseases among humans. In order to establish and maintain infection, the parasite must be able to withstand the hostile environment of the human urogenital tract. This parasite must evade specific immune surveillance mechanisms (Alderete *et al.*, 1991; 1992) and, equally importantly, overcome the constant fluid flow and the mucus barrier bathing the vaginal epithelium. Consequently, cytoadherence by the parasite to the vaginal epithelium (Alderete and Garza, 1985; 1988; Alderete *et al.*, 1988; Arroyo *et al.*, 1992; 1993; Lehker *et al.*, 1991) represents an important property that is essential for initiation and maintenance of infection and fundamental to parasite survival. The cytoadherence mechanism of *T. vaginalis* is complex and probably represents a cascade of reactions, which includes a role for proteinase activity (Arroyo and Alderete, 1989) and signalling of the parasite after initial attachment (Arroyo *et al.*, 1993).

Trichomonal cytoadherence has been shown to be ligand–receptor in nature (Alderete and Garza, 1988; Arroyo *et al.*, 1992; Lehker *et al.*, 1991). Four trichomonad proteins (AP65, AP51, AP33, and AP23) have been identified as the adhesins that specifically mediate attachment to receptors on vaginal epithelial cells (VECs) (Alderete and Garza, 1988; Arroyo *et al.*, 1992). Fulfillment of a number of criteria established that these proteins are authentic adhesins (Arroyo *et al.*, 1992). The relationship between levels of cytoadherence and surface expression of synthesized adhesins was established (Arroyo *et al.*, 1992). Trichomonads expressing low levels of adhesins (Arroyo *et al.*, 1992) have been shown to signal for enhanced synthesis of all adhesins immediately after cytoadherence (Arroyo *et al.*, 1993). Gene expression of the four adhesins was found to be co-ordinately regulated at the transcriptional

level by iron (Lehker *et al.*, 1991), and such regulation may be a mechanism by which the parasite adapts to the constantly changing environment in the vagina. The adhesins are very sensitive to proteinases (Alderete and Garza, 1988; Arroyo *et al.*, 1992) yet, paradoxically, a cysteine–proteinase activity is required for cytoadherence (Arroyo and Alderete, 1989). Finally, the adhesins appear to be immunorecessive, as evidenced by the difficulty of generating high-titered antiserum and monoclonal antibodies (mAbs) in experimental animals (Alderete and Garza, 1988; Arroyo *et al.*, 1992; 1993; Lehker *et al.*, 1991).

In order to explore trichomonal cytoadherence at a more molecular level, the AP65 adhesin was cloned and characterized. The data presented in this paper demonstrate the presence of a multiple-gene family involved in coding for AP65 and further show that this aspect of the host–*T. vaginalis* interrelationship is more complex than initially thought (Arroyo *et al.*, 1992). The significance of our results is discussed below.

Results

Isolation of cDNAs and recombinant AP65

Successful isolation of recombinant *Escherichia coli* colonies, which were immunoreactive with antisera and mAb to the AP65 adhesin (Arroyo *et al.*, 1992), was possible only when mRNA derived from high-iron-grown parasites was used to generate a cDNA library. Two clones, labelled F11.2 and F11.5, contained inserts of sufficient size to encode the full-length adhesin, suggesting that the entire structural gene may be present in these cDNAs. Therefore, these clones were evaluated in greater detail.

Figure 1A shows the four adhesin proteins purified from the established ligand assay (lane 1). Immunoblotting with antiserum to AP65 showed strong reactivity with the trichomonad AP65 protein which bound to fixed HeLa cells (lane 2) (Arroyo *et al.*, 1992), and similar results were obtained with mAb. Figure 1B shows stained total-protein patterns from recombinant *E. coli* without (lane 1) and with F11.2

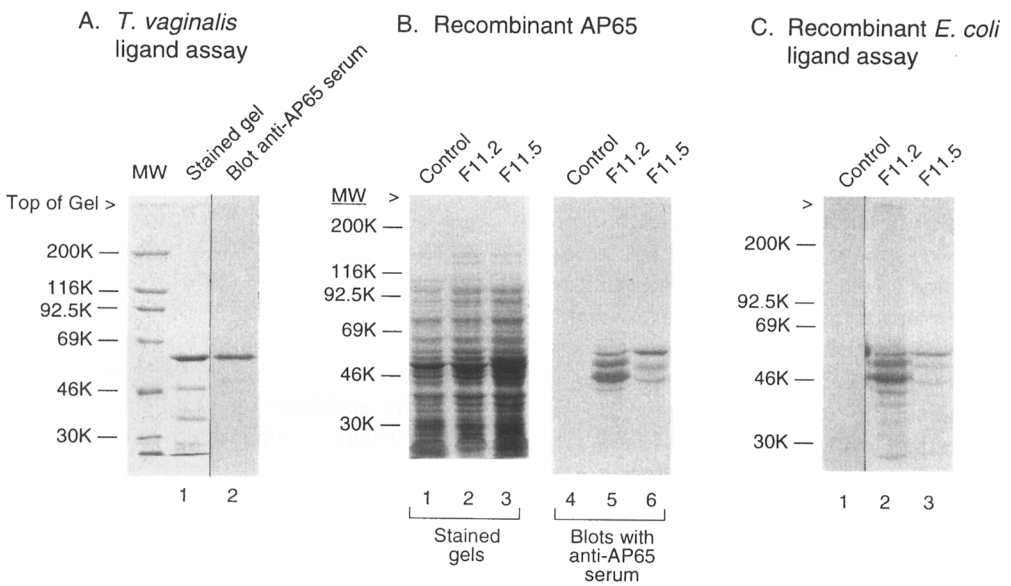

Fig. 1. Ligand-assay analysis of trichomonad adhesins and AP65 (A) and identification of recombinant AP65 proteins expressed in *E. coli* (B) which bind to HeLa cells (C).
A. Trichomonad adhesins were readily detected in the Coomassie brilliant blue-stained acrylamide gel after SDS–PAGE of proteins isolated from fixed HeLa cells by the ligand assay (lane 1). Adhesin AP65 was detected in a duplicate gel after immunoblotting with antiserum to AP65 (lane 2).
B. Coomassie brilliant blue-stained total proteins (lanes 1–3) of lysates of control *E. coli* with vector alone (control) were compared with proteins in lysates of recombinant *E. coli* containing F11.2 and F11.5 cDNAs. Lack of overexpression required immunoblotting (lanes 4–6) for visualization of recombinant proteins recognized by antiserum to AP65. No bands were seen in control *E. coli* lysate (lane 4), and no reactivity with any of the recombinant proteins was ever observed with pre-bleed rabbit serum. Molecular-size markers (Bio-Rad) are shown on the left in kDa.
C. Recombinant proteins expressed by *E. coli* bind to fixed HeLa cells in the ligand assay (see the *Experimental procedures*). After SDS–PAGE and immunoblotting of proteins eluted from fixed HeLa cells, recombinant proteins encoded by F11.2 (lane 2) and F11.5 (lane 3) cDNAs were detected with antiserum to AP65. No bands were seen with antiserum to AP65 when lysate of control *E. coli* harbouring the plasmid without any insert was used in the ligand assay performed simultaneously under identical conditions (lane 1). As above, no detection of proteins was seen with pre-bleed, control serum.

(lane 2) and F11.5 (lane 3) cDNAs. No increase in amounts of protein bands above that of the *E. coli* control was seen, suggesting a lack of overexpression or a high turnover of the protein encoded by these two cDNAs. Nevertheless, bands were seen with antiserum in immunoblots of corresponding recombinant *E. coli* lysates (lanes 5 and 6). Proteins of *E. coli* harbouring the vector without any inserts were used as controls in immunoblots and never reacted with anti-adhesin serum (lane 4). In addition, a pooled preparation of pre-bleed rabbit serum failed to detect any recombinant proteins (data not shown). Detection of multiple bands was not unexpected because of the sensitivity of the trichomonad adhesins to proteinases (Alderete and Garza, 1988; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992) and is consistent with that reported previously for a trichomonad protein expressed in *E. coli* (Dailey and Alderete, 1991).

Finally, both recombinant proteins were capable of binding to fixed HeLa cells in the ligand assay (Fig. 1C). Recombinant proteins were readily detected in immunoblots with rabbit anti-AP65 serum (lanes 2 and 3) but not with control, pre-bleed serum. These results show that the immuno-crossreactive F11.2 and F11.5 recombinant proteins express receptor-binding epitopes for HeLa cell surfaces, as does the trichomonad AP65 protein (Fig. 1A, lane 2).

Molecular sequence analysis and characteristics of AP65 cDNA clones

The nucleotide sequences of the AP65 cDNAs represented by F11.2 and F11.5 were obtained, and inserts were 1736 bp and 1724 bp in length, respectively. Both sequences contained a 1629-nucleotide (nt) open reading frame (ORF) that encoded for a protein of 543 amino acids. Based on the cDNA sequences, the predicted molecular masses of the respective proteins were ~60 700 daltons (60.7 kDa) and 60.6 kDa. As the apparent size of the adhesin (Fig. 1A) was not consistent with that predicted from the sequence and both cDNAs had a 3′ poly-A tail, this indicated that a portion at the 5′ end was missing.

We therefore performed the 5′ ampliFINDER RACE method (see the *Experimental procedures*) in an attempt to obtain additional sequence at the 5′ end. Of importance was the fact that the cDNAs were similar but not identical, with 87% identity at the DNA level and 91% identity at the protein level. This enabled us to identify unique oligonucleotides for the RACE method. This approach was important in order to obtain 5′-end sequence specific for each cDNA. Polymerase chain reaction (PCR) products were derived which gave new sequence information for the 5′ ends. Use of the F11.2 primer resulted in 30 new nucleotides and a 142 nt overlap identical to the F11.2 cDNA sequence. Use of the F11.5 primer gave 40 extra bases with a 100 bp overlap with the F11.5 cDNA sequence. A new translational start site was found for both genes.

The 5′-end PCR products plus the sequences of the F11.2 and F11.5 cDNAs gave full-length sequences (GenBank accession numbers U18346 and U18347, respectively). Both complete sequences coded for proteins of 567 amino acids with predicted molecular masses of 63.3 kDa and 63.1 kDa, respectively, consistent with the known electrophoretic mobility of the adhesin(s) (Fig. 1A). Comparisons between F11.2 and F11.5 sequences showed 87% identity at the nucleotide level and 91% at the amino acid level. Neither of the cDNAs was AT rich; both had an AT/GC ratio of ~1. The predicted proteins encoded by F11.2 and F11.5 had isoelectric points of 7.29 and 8.12, respectively.

Each of the AP65 adhesin genes is distinct and present in multiple copies in the T. vaginalis *genome*

High-resolution mapping (Fig. 2) of both F11.2 and F11.5 cDNAs using various restriction endonucleases showed clear differences, thus confirming the sequence data. These results, and the fact that both cDNAs encoded for recombinant proteins immunoreactive with AP65 antiserum, indicated that the F11.2 and F11.5 cDNAs encoded two distinct AP65 proteins, now called AP65-1 and AP65-2.

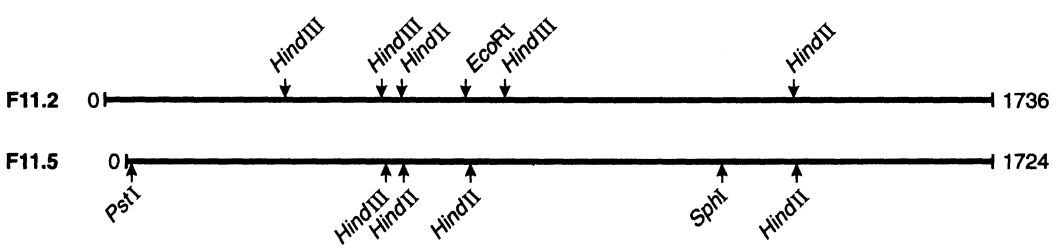

Fig. 2. Restriction analysis confirms that F11.2 and F11.5 cDNAs are different. Arrows represent restriction-endonuclease cleavage sites on the cDNA confirmed experimentally and in accordance with the sequence. The enzymes listed are those used to perform the Southern analysis shown in Fig. 3 and illustrate the differences between the cDNAs.

Southern analysis was performed on genomic DNA treated with cutting enzymes (*Eco*RI for F11.2, and *Pst*I and *Sph*I for F11.5) and non-cutting enzymes (*Pst*I, *Sph*I, *Bam*HI and *Bst*XI for F11.2, and *Bam*HI and *Bst*XI for F11.5), based on the restriction maps (Fig. 2). When non-cutting enzymes for F11.2 and F11.5 were employed, hybridization with either ^{32}P-labelled F11.2 or F11.5 cDNA probes gave multiple bands. As expected, digestion with internal cutting enzymes yielded a greater number of hybridizing bands for the respective probes.

However, as F11.2 and F11.5 cross-hybridized, it became necessary to repeat the experiments using probes specific for each cDNA. This was possible using the specific 5′-end PCR products derived from each cDNA (described above), which had little homology at the sequence level. Figure 3 (part IA) shows the hybridization of each blotted 5′-end PCR product with only the corresponding radiolabelled cDNA from which it was derived. In addition, Southern analysis was performed with plasmid containing F11.2 or F11.5 digested with *Hin*dII or *Hin*dIII and probed with radiolabelled 5′-end PCR products. Hybridization of the nick-translated 5′-end products occurred with the plasmid digested with *Hin*dII, which retained the 5′ end of the homologous cDNA reactive with the 5′-end PCR product (part IB). The small band detected with each PCR product after *Hin*dIII digestion was the expected size of DNA. This small band was derived from the *Hin*dIII sites at the multiple-cloning site of the plasmid (not shown) and within each cDNA (Fig. 2).

Finally, Fig. 3 shows that Southern analysis of the restricted genomic DNA using each of the F11.2 (part IIA) and F11.5 (part IIB) 5′-end PCR products as probes generated multiple bands. The hybridization of each specific 5′-end product with genomic DNA established that both *ap65-1* and *ap65-2* (represented by F11.2 and F11.5 cDNAs, respectively) are present in the *T. vaginalis* genome. Enzymes that neither cut within the 5′-end PCR products nor digest each cDNA (*Bam*HI, *Bst*XI, *Pst*I and *Sph*I for F11.2, and *Bam*HI, *Bst*XI and *Eco*RI for F11.5) gave multiple bands, illustrating the probable multiple-copy nature of both adhesin genes. The different banding patterns reaffirmed the distinct nature of the two AP65 genes.

Only one iron-regulated transcript of the expected size appears for each of the AP65 genes

The isolation of two different and independent cDNA

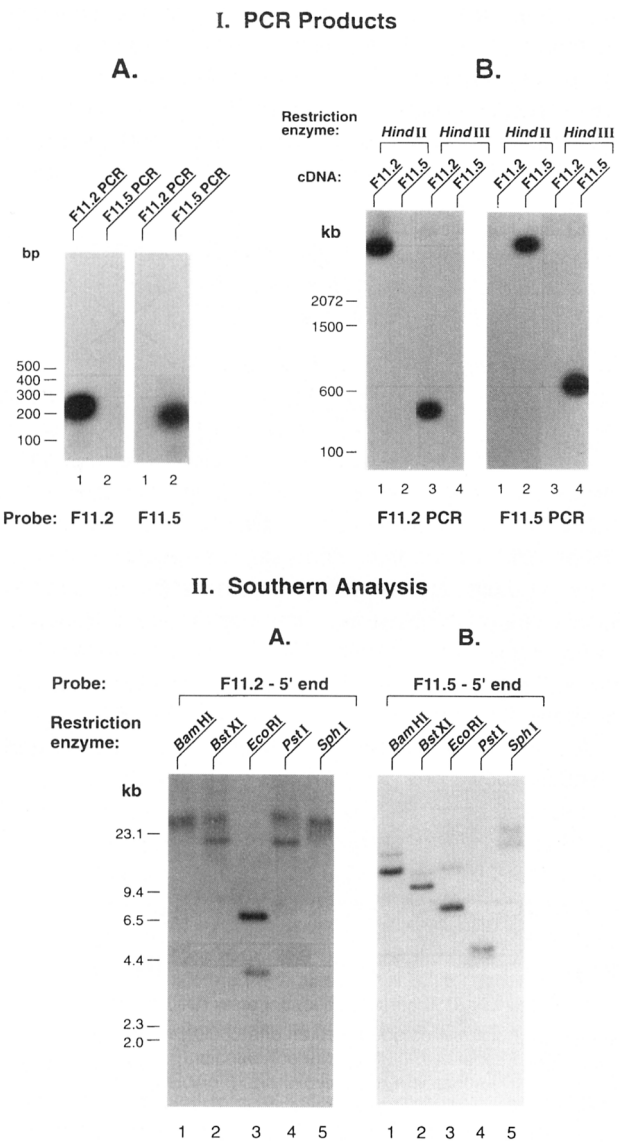

Fig. 3. 5′-end PCR products unique for F11.2 and F11.5 show that *ap65-1* and *ap65-2* have a multiple copy character.
IA. PCR products of the 5′ ends of both F11.2 (lanes 1) and F11.5 (lanes 2) were generated using specific primers (see the *Experimental procedures*) and 5.0 μl of amplified products were electrophoresed through 2% Metaphor agarose gel (FMC BioProducts). Blotting onto the Zeta-probe membrane was followed by hybridization with ^{32}P-labelled cDNA inserts. The 100 bp DNA ladder (Gibco BRL) was used as a molecular-size marker. These results affirm the non-cross-hybridizing nature of the 5′-end PCR products.
IB. Southern analysis of equal amounts of purified plasmid containing the F11.2 and F11.5 cDNA inserts digested with *Hin*dII and *Hin*dIII. Plasmid DNA was electrophoresed in 1% agarose and transferred to the Zeta-probe membrane for hybridization. Duplicate membranes were then probed with either of the radiolabelled, purified 5′-end PCR products (Wizard PCR Preps DNA Purification System, Promega Corp.). The sizes of the bands were as expected considering the restriction map of the cDNA inserts (Fig. 2) and the plasmid (Invitrogen).
II. Southern analysis of restricted genomic DNA probed with ^{32}P-labelled, purified 5′-end PCR products. Procedures, preparation of genomic DNA, and hybridization were as described in the *Experimental procedures*. Note the appearance of two bands when genomic DNA was restricted with non-cutting enzymes (Fig. 2) and probed with F11.2-specific (see IIA) and F11.5-specific (see IIB) 5′-end PCR products. More complex banding patterns were seen when the entire cDNAs were used as probes; this is as expected because the cDNAs cross-hybridized with both genes.

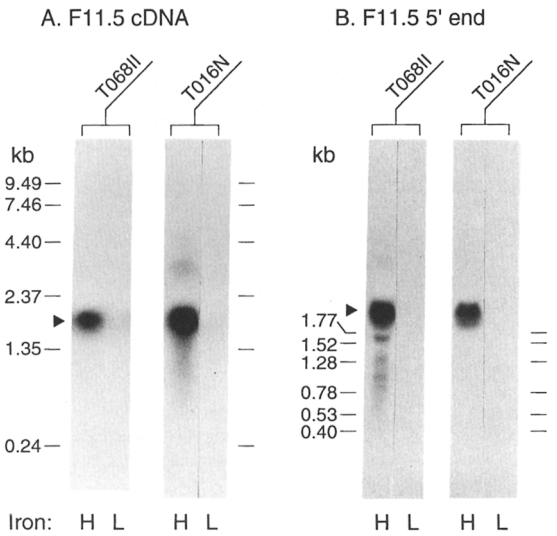

Fig. 4. Iron up-regulates expression of both AP65 genes. Purified total RNA (30 μg) from isolates T016N and T068II grown in iron-replete (H) and iron-limiting (L) medium was electrophoresed in 1% denaturing formaldehyde agarose gels. RNA molecular-size markers (kb) (GIBCO-BRL) were used for size estimation. Hybridization was carried out using ^{32}P-labelled F11.5 cDNA (A) or F11.5 5′-end PCR products (B) as probes. Identical results were obtained with F11.2 cDNA and F11.2 5′-end PCR products. Longer exposure detected a transcript of the same size for the low-iron-grown trichomonads.

clones indicated that the two AP65 genes were transcribed. Northern analysis was performed on RNA isolated from trichomonads. Figure 4 shows transcripts of ~1.8 kb detected in two representative fresh isolates. Radiolabelled F11.5 cDNA (Fig. 4A) or F11.5-specific 5′-end PCR product (Fig. 4B) was used to probe the RNA blots. Detection of the transcript occurred only with total RNA from high-iron-grown trichomonads, as was expected (Lehker *et al.*, 1991). Longer exposures of X-ray film did detect basal levels of transcripts in RNA derived from low-iron-grown organisms. Identical results were obtained when the F11.2 cDNA and F11.2 5′-end products were used as probes. Examination of numerous other isolates also gave similar results. Quantification of total purified RNA and ethidium bromide (EtBr)-stained duplicate gels showed identical amounts of total RNA in all lanes.

5′ sequences reveal leader sequences and putative promoter regions

Twelve amino acids encoded by the cDNAs were not present on the mature protein, as evidenced by N-terminal amino acid sequencing data of the AP65 adhesin purified from the ligand assay (Fig. 5A, to the right of the arrow). This suggested that the AP65 protein was synthesized with a leader sequence that was cleaved to form the mature polypeptide. The N-terminal-sequence data further confirmed the correct codon usage, which was identical to that previously determined for a trichomonad immunogen (Dailey and Alderete, 1991).

The leader sequences and their putative cleavage sites, as shown in Fig. 5A, were found upon analysis of the 5′-end PCR products generated above. Both AP65-1 and AP65-2 leader sequences were very similar in that nine out of 12 residues were identical and the cleaved peptides showed similarities to the signal peptides of other known *T. vaginalis* proteins (Fig. 5B). A comparison of the leader peptides revealed that all of the sequences begin with Met–Leu and have an arginine at the −2 position relative to the cleavage site, consistent with that reported before (Lahti *et al.*, 1992).

The 5′ untranslated regions of the AP65 clones were unusually short (13–17 nucleotides) (Fig. 5C). No typical eukaryotic promoter elements, such as the TATA box, were found. However, an examination of the sequence 5′ to the start site revealed similarity to the promoter elements of other *T. vaginalis* protein-coding genes that have been reported (Quon *et al.*, 1994). Alignment with the 13 bp consensus sequence for *T. vaginalis* promoters showed that between 8–10 bases of each AP65 5′ end conformed to the pattern.

Amino acid sequence analysis reveals that AP65-1 and AP65-2 have possible membrane domains and malic enzyme-like sequences

The hydropathy plots of AP65-1 and AP65-2 (Kyte and Doolittle, 1982) show that the patterns are very similar with only a few differences (data not shown). Computer analysis of the plots revealed five hydrophobic regions in AP65-1 that might be associated with the membrane. Of those five regions, four are present in AP65-2. The identification of at least one possible membrane-spanning segment in both proteins is consistent with the surface localization of AP65 shown before (Alderete and Garza, 1988; Arroyo *et al.*, 1992). Finally, the relative position within the proteins of each membrane-associated domain is shown for both AP65-1 and AP65-2 in Fig. 6A.

A scan of the SWISSPROT (Release 28), EMBL and GenBank (Release 37) databases using the FSTPSCAN and FSTNSCAN programs based on the algorithm of Lipman and Pearson (1985) revealed significant similarity between the AP65 clones and various malic enzymes. When compared to human malic enzyme, for example, both AP65-1 and AP65-2 showed ~54% identity at the nucleotide level and 38% identity at the amino acid level. Although homology was found throughout the proteins, four regions were particularly striking: residues 111–119, 163–196, 265–300, and 312–330. Relative positioning of these regions and comparison with the membrane-associated domains is shown in Fig. 6B. The identity to malic enzyme

A SEQUENCE OF REPRESENTATIVE AP65 5' ENDS

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                  *                                   ↓
                  M  L  T  S  S  V  S  L  P  A  R  E  L  S  R  K  V  L  P  T  L  K  T  G  M
AP65-1  TTTTTGATTAAAGATGCTTACATCTTCAGTCTCTCTTCCAGCACGTGAACTCTCCCGCAAGGTTCTCCCAACCCTCAAGACAGGAATG  88
                  M  L  T  S  S  V  S  V  P  V  R  N  I  C  R  A  K  V  P  T  L  K  T  G  M
AP65-2  TTTTCAGATTAAAGATGCTCACATCTTCAGTCTCTGTTCCAGTCCGCAACATCTGCAGGGCTAAGGTCCCAACCCTCAAGACAGGCATG  89

           T  L  L  Q  D  G  D  L  N  K  G  T  A  F  T  K  E  E  R  D  R  F  N  L  R  G  L  L
AP65-1  ACCTTACTTCAGGATGGAGATCTCAACAAAGGTACAGCTTTCACAAAAGAAGAACGTGACCGCTTCAATCTTCGTGGCCTCCTC  172
           T  L  L  Q  D  G  D  L  S  K  G  S  A  F  T  K  E
AP65-2  ACACTCCTTCAGGATGGTGATCTTTCCAAGGGTTCTGCCTTCACAAAGGAG  140
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B COMPARISON OF PUTATIVE SIGNAL PEPTIDES FOR AP65 AND OTHER *T. VAGINALIS* PROTEINS

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AP65-1    M L T S S V S L P A R E ↓
AP65-2    M L T S S V S V P V R N ↓

β-SCS53   M L S S S F A R N ↓
β-SCS1    M L S A S S N F A R N ↓
α-SCS1    M L A G D F S R N ↓
α-SCS2    M L S S S F E R N ↓
Fd        M L S Q V C R F ↓
AK        M L S T L A K R F ↓
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C PUTATIVE PROMOTER ELEMENTS OF AP65 CLONES AND OTHER *T. VAGINALIS* GENES

AP65 clones		# agreeing residues
	F11.2-C1 — T T T T G A T T A A A G A T G	9
	F11.2-C13 — T T T T T T T G A T T A A A G A T G	10
	F11.5-C1 — T T T T C A G A T T A A A G A T G	8
	F11.5-C2 — T T T T C A G A T T A A A G A T G	8
	F11.5-C6 — T T T T C A G T T T C A G A T T A G A T G	8
	F11.5-C5 — T T T T T A G A T T A A A G A T G	8
	F11.5-C10 — T T T T T A G A T T A A A G A T G	8
	FD. — T A C T T C A C T T C T C T T A G C G A A T G	12
	β-SCS — T T G A T C A C T T C A C A T T A C A A T G	12
	α-SCSB — T T G T T C A C T T C A C A T T A A T G	12
	α-TUB — A G T G T C A C T C T T C A T C A A T G	11
	cHSP70 — C A T C T C A T T T T T T A A T A A T G	11
	Pgp1 — C A G A C C A T T A A T C A T T A G T G A T G	11
	β-TUB — A A T A T C A T T A T T C A C A T G	10
	CONSENSUS — T C A Y T W Y T C A T T A	13

Fig. 5. Molecular analysis of nucleotide and amino acid sequence of the 5′ ends of the adhesin genes and proteins.
A. Nucleotide sequence of representative AP65 5′-end clones of both AP65 genes obtained by RACE. Underlined bases indicate new sequence information. Numbers refer to nucleotide positions. The predicted amino acid sequence is shown above the DNA sequence. An asterisk denotes the translational start site (ATG). The signal peptide is in bold and the arrow represents its putative cleavage site.
B. Comparison of the AP65 putative signal peptides with the leader sequences of *T. vaginalis* hydrogenosomal proteins β-succinyl CoA synthetase 53 (β-SCS53) and β-SCS1 (Johnson *et al.*, 1993; Lahti *et al.*, 1992), α-succinyl CoA synthetase 1 (α-SCS1) and α-SCS2 (Lahti *et al.*, 1994), ferredoxin (Fd) (Johnson *et al.*, 1990), and adenylate kinase (AK) (Länge *et al.*, 1994). The arrow denotes the cleavage sites determined by N-terminal sequencing, which was also performed on the AP65 adhesins (see the *Experimental procedures*).
C. Comparison of the putative promoter elements of the AP65 clones and other *T. vaginalis* genes. The 5′ sequences immediately preceding the ATG start site are shown for representative AP65 5′-end clones and for other protein-coding *T. vaginalis* genes. Abbreviations: Fd, ferredoxin; β-SCS, β-succinyl CoA synthetase; α-SCSB, α-succinyl CoA synthetase; α-TUB, α-tubulin; cHSP70, 70 kDa heat-shock protein; Pgp 1, P-glycoprotein 1; and β-TUB, β-tubulin (Quon *et al.*, 1994). The consensus pattern, as defined by Quon *et al.* (1994), is shown below the sequences. The conserved region is boxed and shaded residues follow the consensus sequence.

within these four areas was as high as 78%. In these conserved domains, AP65-1 and AP65-2 were almost identical with only three amino acid differences in total.

Sequence analysis revealed that three of the regions correspond to the dinucleotide-binding sites found in malic enzymes and other enzymes (Fig. 7). The region between amino acids 111–119 shows homology to the putative NADP-binding sites of human and murine malic enzyme, goose fatty-acid synthetase, and human glyceraldehyde 3-phosphate dehydrogenase (Fig. 7A). Residue 120 in both AP65 proteins is a cysteine amino acid conserved among all malic enzymes; this residue is believed to be the malate-binding site (Satterlee and Hsu, 1991). As modification of the cysteine impairs binding of L-malate, but not NADPH, any involvement of this region in binding NADP is unlikely (Satterlee and Hsu,

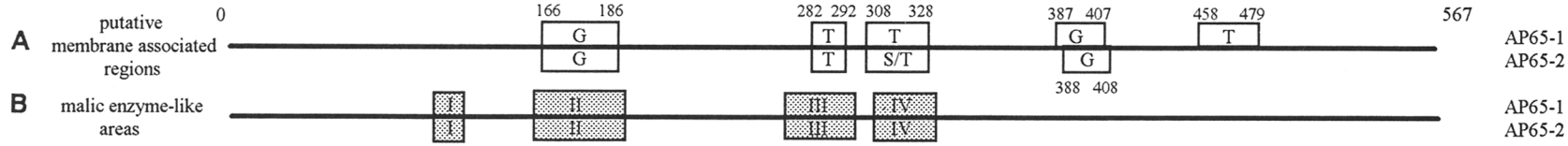

Fig. 6. Positioning of possible membrane-spanning domains and malic enzyme-like domains revealed by amino acid sequence analysis.
A. Predicted membrane-associated areas for each adhesin are indicated. The segments were classified as being globular (G), transmembrane (T), and/or surface located (S) (Eisenberg *et al.*, 1984; Mohana-Rao and Argos 1986; Klein *et al*, 1985). Numbers refer to the positioning of the predicted amino acids from the sequence.
B. Regions that show strong homology to malic enzyme based on amino acid similarities are indicated. These domains include the NADP-binding region (I), the βαβ-ADP binding fold (II), the Mn^{2+}-binding site (III), and the NADP βαβ-binding fold (IV). Sequence comparisons are shown in Fig. 7.

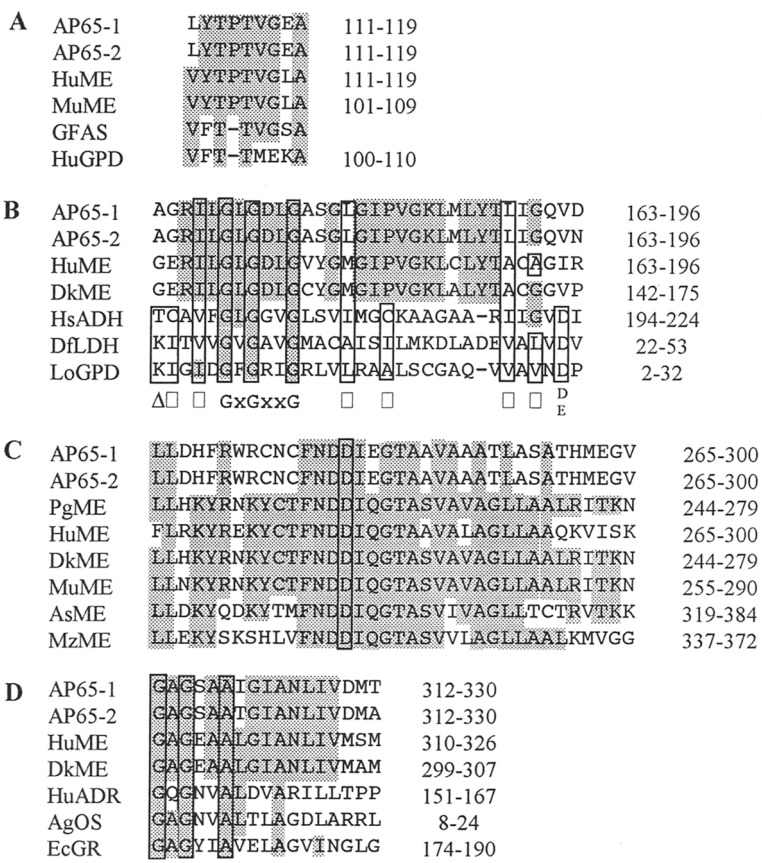

Fig. 7. Amino acid sequence homology between the AP65 clones and conserved regions of several enzymes. For each protein, the sequence and amino acid position of the homologous region are given. Identical amino acids are shaded.
A. Comparison of the AP65 clones with the putative dinucleotide-binding regions of human malic enzyme (HuME), murine malic enzyme (MuME), goose fatty-acid synthetase (GFAS), and human glyceraldehyde-3-phosphatase (HuGPD) (Loeber *et al.*, 1991; Bagchi *et al.*, 1987; Poulose and Kolattukudy, 1983).
B. Sequence homology between AP65 and the putative ADP-binding βαβ folds of HuME, duck malic enzyme (DkME), horse alcohol dehydrogenase (HsADH), dogfish lactate dehydrogenase (DfLDH), and lobster glyceraldehyde phosphate dehydrogenase (LoGPD) (Loeber *et al.*, 1991; Hsu *et al.*, 1992; Wierenga *et al.*, 1985). The consensus sequence, as proposed by Wierenga *et al.* (1985), is shown beneath the sequences. Δ indicates hydrophilic residues, and □ indicates hydrophobic residues. Boxed amino acids adhere to the fingerprint pattern.
C. Alignment of the AP65 clones with the putative Mn^{2+}-binding domains of various malic enzymes. The aspartate residue identified as one of the Mn^{2+}-binding ligands of pigeon-liver malic enzyme (PgME) is boxed. The malic enzymes are: PgME, HuME, DkME, MuME, and that from *Ascaris suum* (AsME), and maize (MzME) (Wei *et al.*, 1994; Loeber *et al.*, 1991; Hsu *et al.*, 1992; Bagchi *et al.*, 1987; Kulkarni *et al.*, 1993; Rothermel and Nelson, 1989).
D. Comparison of the amino acid sequences of AP65 with the NADP-binding folds of HuME, DkME, human adrenodoxin reductase (HuADR), *Agrobacterium* octopine synthase (AgOS), and *E. coli* glutathione reductase (EcGR) (Loeber *et al.*, 1991; Hsu *et al.*, 1992; Scrutton *et al.*, 1990).

1991). A segment spanning residues 163–196 matched a number of elements from the consensus sequence for the ADP-binding βαβ fold (Wierenga *et al.,* 1985; Fig. 7B). The AP65 sequences contain the essential GXGXXG motif prerequisite for NAD-binding. AP65 also shows homology to the NADP-specific βαβ binding fold (Scrutton *et al.*, 1990). Amino acids 312–330 of AP65-1 and AP65-2 include the GXGXXA sequence necessary for the binding of NADP in other proteins (Fig. 7D). Finally, residues 265–300 correspond to the putative Mn^{2+}-binding site of malic enzymes (Wei *et. al.*, 1994) (Fig. 7C), including the highly conserved aspartic acid believed to be one of the Mn^{2+} ligand sites of malic enzyme. It is noteworthy that the amino acid positions of all these regions are similar for the AP65 clones and the malic enzymes. The fact that three of the predicted membrane-associated regions correspond with malic enzyme putative dinucleotide- and metal-binding domains suggests that these hydrophobic segments may not necessarily assume transmembrane properties. Future work will clarify the function of these areas.

Functionality of recombinant adhesins

The strong homology between AP65-1, AP65-2, and malic enzymes raised questions as to the possible role of malic-enzyme activity in *T. vaginalis* cytoadherence. In order to determine whether malic enzyme influenced *T. vaginalis* cytoadherence, it was important to perform a ligand assay with malic enzyme and recombinant proteins AP65-1 and AP65-2.

A competition experiment was performed with recombinant *E. coli* lysates and ^{35}S-labelled adhesins of *T. vaginalis*. Pretreatment of HeLa cells with lysates of *E. coli* expressing each recombinant protein decreased the amount of radiolabelled *T. vaginalis* adhesin binding to host cells (Fig. 8A). Binding of ^{35}S-labelled trichomonad adhesin was not similarly inhibited when HeLa cells were first treated with the same amounts of protein from control *E. coli* lysate. Similar competition experiments were carried out using increasing amounts of commercially available malic enzyme. No inhibition of ^{35}S-labelled trichomonad AP65 was evident when excess (up to 120 μg) purified malic enzyme (Fig. 8A) was first used to pre-treat cells or in co-incubation experiments. In addition, malic enzyme at high concentrations did not inhibit binding of recombinant AP65-1 or AP65-2 to HeLa cells (data not shown). Activity of malic enzyme was monitored throughout to ensure the conditions did not denature the protein.

A mAb (designated F11) previously generated against AP65 purified from the ligand assay (Arroyo *et al.,* 1992) was found to be immuno-crossreactive with malic enzyme. As seen in Fig. 8B, immunoblots with mAb F11 of malic enzyme (lane 1) and recombinant *E. coli* lysates (lanes 2 and 3) revealed recognition of an epitope common to malic enzyme and the adhesins. This same mAb was ineffective at inhibiting *T. vaginalis* organisms attaching to HeLa cells in monolayer cultures (Fig. 8, part C2). In contrast, monospecific rabbit antiserum to AP65 blocked cytoadherence (Fig. 8, part C1) to levels which had been achieved previously (Arroyo *et al.*, 1992).

Finally, commercially purchased malic enzyme was used in a competition experiment in a cytoadherence assay (Arroyo *et al.*, 1992). In data not shown, no decrease in levels of trichomonal attachment to HeLa cells was ever observed when pure malic enzyme, or other serum proteins, such as albumin, used as controls (Arroyo *et al.*, 1992), were added to the co-incubation medium in excess amounts. Only the addition of IgG derived from rabbit anti-AP65 serum readily inhibited adherence, as shown by Arroyo *et al.* (1992).

The AP65 adhesins appear unique to T. vaginalis

It was of interest to examine whether the AP65 adhesins and genes of *T. vaginalis* were possibly present within the genome of other *Trichomonas* species. A ligand assay was performed simultaneously with a cross-hybridization experiment using the cDNA inserts as probes. As a control for the hybridization experiment, duplicate gels of DNA were stained with EtBr after electrophoresis to ensure that identical amounts of DNA were in each lane. Total undigested DNA, purified similarly for all species, was electrophoresed and blotted. Only *T. vaginalis* DNA readily gave a strongly hybridizing band when compared with equal amounts of undigested genomic DNA of *Pentatrichomonas hominis*, *Tritrichomonas suis*, and *Tritrichomonas foetus* (data not shown). These results were seen at various levels of stringency.

The absence of any AP65-like proteins in these species was further demonstrated by the lack of detection of proteins that bound to HeLa cells when immunoblots were screened with anti-adhesin antiserum (data not shown). Under no circumstances were proteins immunocrossreactive with anti-AP65 serum detected for the other species, even when 10-fold greater amounts of extracts were used in the ligand assay for blotting with anti-AP65 antiserum (data not shown).

Discussion

Earlier work had shown that iron-regulated cytoadherence and synthesis of adhesins for *T. vaginalis* (Lehker *et al.*, 1991). It was predictable, therefore, that detection of transcripts encoding the adhesins in Northern blots readily occurred only in mRNA from high-iron-grown parasites. The isolation, by immunoscreening, of cDNAs encoding

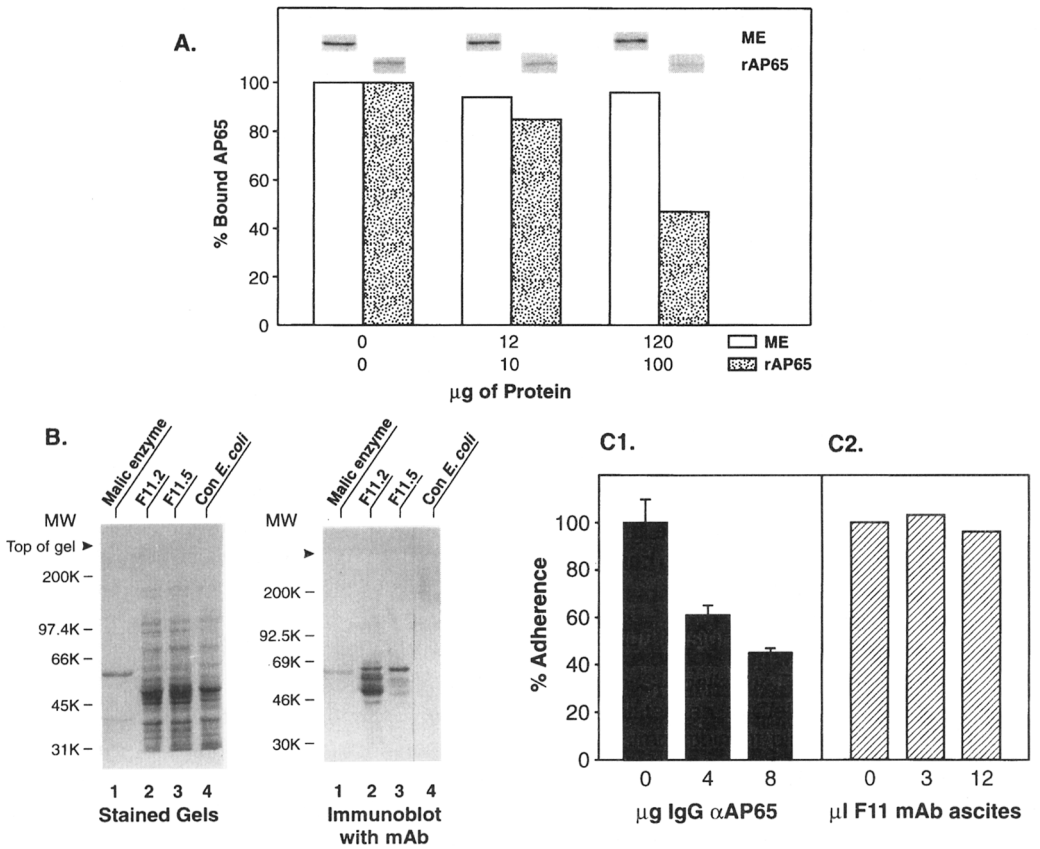

Fig. 8. Adhesins AP65-1 and AP65-2 express biofunctionality and have receptor-binding epitopes unique from malic enzyme-like sequences.
A. Recombinant protein, but not malic enzyme, inhibits binding of ^{35}S-labelled trichomonad AP65 to host cells. Fluorographs of the competition experiment are shown above the bar graph. In the ligand assay, trichomonad AP65 from ^{35}S-labelled *T. vaginalis* extracts was added to fixed HeLa cells, which were first pretreated with either malic enzyme (ME) or extracts of recombinant *E. coli* expressing AP65 from the F11.2 cDNA. Only the recombinant AP65 from *E. coli* extracts competed with ^{35}S-labelled trichomonad adhesin, as shown in the corresponding densitometry results. Increasing amounts of purified ME (clear box) did not inhibit the binding of *T. vaginalis* AP65 to HeLa cells. In contrast, the addition of recombinant AP65 (spotted box) inhibited the binding of trichomonad AP65 in a concentration-dependent manner. As shown in Fig. 1B, 100 μg of *E. coli* lysate containing recombinant adhesin (rAP65) decreased parasite AP65 binding to fixed HeLa cells by ~55%.
B. mAb F11 (Arroyo *et al.*, 1992) reacts with ME (lane 1) and recombinant adhesins encoded by F11.2 and F11.5 cDNAs (lanes 2 and 3), but not with *E. coli* extract containing plasmid without inserts (Con *E. coli* lane). Coomassie brilliant blue-stained gels (left) were compared with immunoblots of duplicate gels (right). The mAb reactions with recombinant adhesins were identical to those seen earlier with antiserum to AP65 (Fig. 1B).
C. mAb F11 to AP65, immuno-crossreactive with ME, does not inhibit cytoadherence of *T. vaginalis* to HeLa cells (C2). Conditions for cytoadherence of isolate T016 were identical to those used by Arroyo *et al.* (1992). Levels of inhibition seen with the antiserum to AP65 were similar to those reported previously (Arroyo *et al.*, 1992) (C1). Ascites of F11 mAb had a concentration of antibody exceeding 1 mg ml^{-1} of specific anti-AP65 IgG antibody.

AP65 was successful only when mRNA from *T. vaginalis* grown in medium supplemented with iron was used to construct the cDNA library. This fact alone shows the importance of understanding the environmental cues which ensure expression of virulence factors prior to attempts at cDNA cloning of relevant genes from this and other pathogenic protozoa.

cDNAs were isolated from an expression library that encoded epitopes crossreactive with antiserum against an adhesin protein called AP65 (Arroyo *et al.*, 1992). This protein is one of four adhesins of *T. vaginalis* important for adherence to host epithelial cells (Alderete and Garza, 1988; Alderete *et al.*, 1988; Arroyo *et al.*, 1992). Here we report the characterization of two cDNAs, F11.2 and F11.5, which appeared to be full length on the basis of size of DNA and the electrophoretic mobilities of the recombinant protein (Arroyo *et al.*, 1992). Restriction mapping (Fig. 2) and Southern analysis of cDNA inserts (Fig. 3) provided evidence that the two cDNAs were distinct. That two discrete genes encoding AP65, now designated *ap65-1* and *ap65-2,* are present within the genome, each in multiple copies, was verified by using highly specific, non-cross-hybridizing 5′-end PCR products generated to both F11.2 and F11.5 cDNAs (Fig. 3).

The F11.2 and F11.5 cDNAs, therefore, code for distinct immuno-crossreactive recombinant proteins, AP65-1 and AP65-2, with properties similar to the *T. vaginalis* AP65 adhesin (Alderete and Garza, 1988; Arroyo *et al.,* 1992). The fact that recombinant proteins bound to HeLa cells (Fig. 1C) allowed us to demonstrate competition of recombinant proteins with trichomonad adhesins for host cell surfaces (Fig. 8A). This latter result shows that both AP65-1 and AP65-2 contain a receptor-recognition site, a finding important for epitope mapping of the recombinant proteins. Whether AP65-1 and AP65-2 interact with the same or a different receptor, the existence of which has been shown (Arroyo *et al.*, 1992), awaits further experimentation.

Iron is clearly an important signal to *T. vaginalis* (Lehker *et al.*, 1991; Lehker and Alderete, 1992), and iron-limited organisms provided with iron increase transcription and synthesis of adhesins (Lehker *et al.,* 1991). In addition, immediately after contact with host cells, the parasites undergo a dramatic morphological transformation concomitant with the rapid synthesis of adhesins (Arroyo *et al.,* 1993). The presence of multiple copies of AP65 genes may ensure the expression and synthesis of adequate amounts of adhesins within a short period of time after contact. It is also possible that the different AP65 genes, although co-ordinately regulated by iron, respond to yet other undefined environmental signals. It is conceivable, for example, that the genes for AP65-1 or AP65-2, but not both, are directly involved in transcription and expression of the adhesin following contact with the epithelial cell surface (Arroyo *et al.,* 1993). The other gene may be responsive at times other than contact, for instance when different amounts and types of iron sources are available in the vagina, such as during menstruation. Future experiments will explore the arrangement and regulation of these adhesin genes.

The deduced amino acid sequences for AP65 and AP65-2 contain putative leader peptides that show similarity to the signal sequences of mitochondrial and hydrogenosomal proteins (Lahti *et al.*, 1992; von Heijne *et al.*, 1989). These signal sequences do not appear to be typical of proteins translocated to the membrane (von Heijne, 1985). We cannot exclude, however, the possibility that these signals, although shorter, are capable of directing the adhesins to the plasma membrane. As several surface and exported proteins from other organisms also do not have typical N-terminal signal peptides (Lottenberg *et al.*, 1992; Joe *et al.*, 1994), the hydrophobic C-termini of the AP65 adhesins may play a role in localizing the molecules to the *T. vaginalis* surface. Alternatively, taking into account that *T. vaginalis* has four adhesins that are co-ordinately expressed, one might envisage that all four proteins are placed within a vesicle and exported in this manner. This possibility would allow for efficient simultaneous surface localization of the four adhesins. Although the exact inter-relationship between the adhesins is not yet known, the fact that each is essential for cytoadherence (Alderete and Garza, 1985; 1988; Arroyo, *et al.*, 1992) reinforces the idea that such co-ordinated expression might necessitate some form of packaging within organelles.

A database search for homologous proteins revealed significant sequence similarity to malic enzymes isolated from a variety of sources. Malic enzyme found in *T. vaginalis* hydrogenosomes normally functions as a metabolic enzyme catalysing the oxidative carboxylation of malate to pyruvate (Markos *et al.*, 1993). It has not been possible to test whether the AP65-1 and AP65-2 adhesins express malic enzyme activity because of the low levels of expression and degradation of recombinant proteins in *E. coli.* This will require the generation of antibodies which are specific for each adhesin but do not crossreact with malic enzyme. These antibody reagents will be useful for enrichment, such as by affinity chromatography, of each adhesin for performing malic enzyme assays and for localization of the molecules within the parasite.

Recent reports have described metabolic enzymes on microbial surfaces (Camara *et al.*, 1994; Joe *et al.*, 1994), and such surface enzymes possess multiple functions, including adherence (Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992; 1993; Vacca-Smith *et al.*, 1994). Some of the earliest reports of enzymes having important roles beyond those in metabolism 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). Interestingly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found on the surface of group A streptococci where it functions as a plasmin receptor (Lottenberg *et al.*, 1992, Pancholi and Fischetti, 1992). This enzyme has also been identified as a major immunogen on the outer membrane of *Schistosoma mansoni* (Goudot-Crozel, 1989). A number of alternative functions have been described for GAPDH, including protein kinase activity (Kawamoto and Caswell, 1986), bundling of microtubules (Huitorel and Pantaloni, 1985), uracil DNA glycosylase activity (Meyer-Siegler *et al.,* 1991), and binding of fibronectin, lysozyme, and cytoskeletal proteins (Pancholi and Fischetti, 1992). A surface-expressed α-enolase has also been implicated as a plasminogen receptor (Miles *et al.*, 1991). Another surface-localized enzyme, glucosyltransferase, has been demonstrated to mediate the adhesion of *Streptococcus gordonii* to human endothelial cells (Vacca-Smith *et al.*, 1994).

Malic enzyme is widely distributed among plants, bacteria and animals and has a variety of metabolic roles. It has been found in several forms, including cytoplasmic, mitochondrial and, interestingly, membrane-bound forms

(Kendrick and Ratledge, 1992). In some instances, the location of a protein may play a role in its function. One way of regulating protein structure and its function is via ADP-ribosylation (Pancholi and Fischetti, 1993). In the case of GAPDH, the surface form of the enzyme, but not the cytosolic form, is capable of auto-ADP-ribosylation (Pancholi and Fischetti, 1993). This process decreased enzymatic activity without affecting the binding properties of the surface enzyme. The cytosolic form remained catalytic without ADP-ribosylation. This possibility requires testing in the future for our malic enzyme-like adhesin proteins.

These data do not exclude the possibility that one of the AP65 proteins represents the trichomonad malic enzyme. AP65-1 and AP65-2 share with malic enzymes, three putative dinucleotide-binding domains, the malic enzyme metal-binding sequence, and the residue believed to be the malate-binding site. Nevertheless, neither purified commercially available malic enzyme nor extracts of control *E. coli* containing malic enzyme interfered with *T. vaginalis* cytoadherence to host cells, showing a lack of involvement of enzymatic activity in adherence. Furthermore, purified malic enzyme did not prevent binding of the trichomonad AP65 to HeLa cells in a ligand assay. Equally noteworthy, a mAb that is immuno-crossreactive with the adhesins and malic enzyme also failed to inhibit *T. vaginalis* cytoadherence. These data suggest strongly that, regardless of whether the adhesins possess malic enzyme-like domains, the receptor-binding epitope(s) for the AP65 adhesins is specific and unique to malic enzymes.

Furthermore, because of the highly conserved nature of malic enzymes and the malic enzyme sequences in the adhesins, the placement of host-like proteins on the surface of this sexually transmitted agent may be significant and play a role in immune evasion. This would represent a form of molecular mimicry — something that has received attention as an important mechanism by which parasites escape recognition by the host immune system (Damian 1989). We have previously reported on the immunorecessive nature of the adhesins, and preliminary data suggest that antibody to the adhesins, if it exists, is at low levels in human serum or vaginal wash (Alderete *et al.*, 1991; 1992). This strategy by the parasite has important implications for vaccine development using biofunctional trichomonad molecules, such as adhesins, which mimic host proteins. Future identification of the receptor-binding epitope and the extent of similarity of this epitope with host sequences will be important in the consideration of these proteins as vaccine candidates. Finally, this work on one of the four adhesins will serve as a model for molecular characterization of the genes and proteins of the other three adhesins (Arroyo *et al.*, 1992).

Experimental procedures

Microorganisms and culture conditions

T. vaginalis T016N and T068II were fresh clinical isolates that expressed high amounts of adhesins under iron-replete growth conditions (Arroyo *et al.*, 1993; Lehker *et al.*, 1991). The growth medium of Trypticase–yeast extract–maltose with 10% heat-inactivated horse serum (Diamond, 1957) was supplemented, for iron-replete conditions, with 250 μM ferrous ammonium sulphate-hexahydrate (Sigma), which was prepared as a 100-fold stock solution made in 50 mM sulphosalicylic acid (Lehker *et al.*, 1991). Parasites were grown to the late-logarithmic phase of growth. Isolates of other trichomonad species, including *P. hominis* (ATCC 30000, American Type Culture Collection), *T. suis* (ATCC 30167), and *T. foetus* KV1 (Alderete, 1983), were handled similarly.

Recombinant *E. coli* INVαF′ (Invitrogen Corp.) harbouring the phagemid vector pcDNAII (Invitrogen) with the cDNA clones F11.2 or F11.5, which code for the AP65 adhesin, were cultured in Luria–Bertani (LB) broth or LB agar plates with 60 μg ml^{-1} ampicillin (Sambrook *et al.*, 1989).

Isolation of nucleic acids from T. vaginalis

Total RNA from high-iron-grown parasites was isolated by the procedure of Chomczynski and Sacchi (1987) using acid phenol. Briefly, 10^8 parasites were washed and immediately lysed by addition of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 100 mM 2-mercaptoethanol). Then, 2 M sodium acetate pH 4.0, acid phenol, and a chloroform–isoamyl alcohol mixture were added sequentially (Chomczynski and Sacchi, 1987) and the lysate mixed by inversion after each addition. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10 000 × *g* for 20 min at 4°C. After centrifugation, RNA in the aqueous phase was precipitated by ethanol. The RNA pellet was dissolved in solution D, reprecipitated and rinsed in ethanol before dissolving in 0.5% SDS at 65°C for 10 min. RNA was stored at −70°C. The mRNA was purified with oligonucleotide(dT)-cellulose type 7 (Pharmacia LKB Biotechnology).

Total genomic DNA from trichomonads was isolated by standard procedures. Briefly, 10^8 parasites were lysed with 0.2% SDS in buffer consisting of 100 mM NaCl, 10 mM EDTA and 10 mM Tris-HCl, pH 8.0 before extraction four times using a phenol–chloroform mixture. After two further chloroform extractions, DNA was precipitated by ethanol. The DNA pellet was resuspended and incubated in TE buffer (1 mM EDTA and 10 mM Tris-HC1, pH 8.0) containing 200 μg ml^{-1} of RNase A for 10 min at 37°C. Then, proteinase K (100 μg ml^{-1} final concentration) was added prior to incubation for 45 min at 37°C. DNA was phenol–chloroform extracted and precipitated again by ethanol and dissolved in TE buffer for storage at 4°C.

Generation and isolation of cDNAs

A *T. vaginalis* cDNA expression library was constructed by directional cloning in the pcDNAII phagemid vector, as recommended by the manufacturer (Invitrogen). Recombinant

colonies were screened (Sambrook *et al.*, 1989) with rabbit antiserum and mAb raised against the purified AP65 adhesin (Arroyo *et al.*, 1992; 1993; Lehker *et al.*, 1991). Antiserum was adsorbed with *E. coli* lysate before screening. Two cDNAs obtained from immunoreactive colonies were isolated and purified. Recombinant plasmids were recovered by alkaline lysis (Invitrogen) as recommended by the manufacturer. Insert analysis was performed by treatment of plasmids with *Xma*III and subsequent electrophoresis of DNA on 1% agarose gels in TAE buffer (1 mM EDTA in 40 mM Tris-acetate, pH 8.0) (Sambrook *et al.*, 1989).

DNA sequencing and analysis

DNA sequencing was performed by the dideoxy chain-termination method using the Sequenase 2.0 DNA Sequencing kit (United States Biochemical) as recommended by the manufacturer. Sequence information was obtained by stepwise sequencing. Initially, universal and reverse M13 primers (Promega) were used. As new sequence data became available, cDNA-specific oligonucleotides were synthesized (Center for Advanced DNA Technologies, University of Texas Health Science Center (UTHSCSA), and Genosys Biotechnologies) and utilized to continue the sequence determination. Both strands of the cDNA were sequenced at least three times. Any problem areas or compressions were resolved by substituting 7-deaza-dGTP or dITP for dGTP. Computer analysis of the sequence was carried out using PC/GENE (Release 6.8) (IntelliGenetics, Inc.).

Nucleotide sequence accession numbers

The nucleotide sequences of *ap65-1* and *ap65-2* have been assigned GenBank accession numbers U18346 and U18347, respectively.

Rapid amplification of F11.2 and F11.5 cDNA 5′ ends

The 5′-ampliFINDER RACE (rapid amplification of cDNA 5′ ends) kit (Clontech Lab, Inc.) was used as recommended by the manufacturer to generate highly specific probes for differentiation of the AP65 genes. Partially purified mRNA (5 μg) was used for first-strand cDNA synthesis, which was performed by priming the RNA with an oligonucleotide (P1) common to both F11.2 and F11.5 cDNA clones. The oligonucleotide P1 represented AP65 antisense nucleotides with the sequence 5′-GCGGATACGAGCAGCTTGTTCATCC-3′. An anchor with an *Eco*RI restriction site (Clontech) was then ligated to the first-strand cDNA. Amplification was performed using the anchor primer (Clontech) and the second oligonucleotide (P2) representing the internal AP65 antisense sequence of F11.2 containing two internal *Eco*RI sites (5′-GAATTCGAATTCGAGGAGGCCACGAAGATTGAAGCGG-3′) and of F11.5 without *Eco*RI sites (5′-CTCCTTTGTGAAGGCAGAACCC-3′). Using the P2 primers, the F11.2- and F11.5-specific cDNA sequences were amplified during 35 cycles of the PCR at 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, with a final extension time of 7 min (Clontech). The amplified products were analysed on Metaphor agarose gels (FMC BioProducts) and subcloned directly into pCRII (Invitrogen). The existence of primer sequences and F11.2- and F11.5-specific sequences was confirmed by cross-hybridization experiments of PCR products as shown in the *Results* (Fig. 3, A and B). Both strands of the PCR products were sequenced at least three times using universal M13 primers and sequence-specific oligonucleotides.

Southern and Northern blot analysis

For Southern analysis (Southern, 1979), cDNA or trichomonad genomic DNA digested with specific restriction enzymes was transferred to a Zeta-probe membrane (Bio-Rad) as recommended by the manufacturer. Hybridizations were carried out using purified, representative nick-translated cDNA inserts used as probes (Sambrook *et al.*, 1989). Blots were first treated in prehybridization solution (50% formamide, 120 mM Na_2HPO_4, 250 mM NaCl, 7% SDS and 1 mM EDTA) at 42°C. Radiolabelled probes were then added to new prehybridization solution and incubated with the blots for 18 h at 42°C. Filters were then washed sequentially with 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0)–0.1% SDS, 0.5× SSC–0.1% SDS and 0.1× SSC–0.1% SDS at 42°C for 30 min each wash. Blots were then exposed to Kodak XRP-5 X-ray film.

Transcripts were detected by performing Northern blot analysis of electrophoresed total trichomonad RNA on 1% agarose in 2 M formaldehyde gels (Sambrook *et al.*, 1989) that was transferred to a Zeta-probe membrane (Bio-Rad). Blots were probed with nick-translated cDNA inserts from each adhesin. Hybridization reactions were performed as for Southern analysis and as described previously by Khoshnan and Alderete (1993; 1994).

SDS–PAGE and immunoblotting of recombinant proteins and malic enzyme

Overnight (o/n) cultures of recombinant *E. coli* (1 ml) were pelleted and suspended in 200 μl of electrophoresis sample buffer (Laemmli, 1970) and boiled for 3 min (Sambrook *et al.*, 1989). Chicken liver malic enzyme (EC 1.1.1.40) (Sigma) was diluted in electrophoresis sample buffer to a final concentration of 0.25 μg μl^{-1}. Aliquots of 10 μl of recombinant *E. coli* lysates and diluted malic enzyme were subjected to SDS–PAGE using 10% separating and 4% stacking gels (Alderete and Garza, 1985; Alderete *et al.*, 1986a,b). Following electrophoresis, gels were stained with Coomassie brilliant blue. Duplicate gels were transferred to nitrocellulose (NC) for immunoblotting (Towbin *et al.*, 1979).

The NC blots were rinsed with TNT buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween 20) and blocked with TNT buffer containing 5% skimmed milk (BLOTTO) for 1 h at room temperature (RT) (Sambrook *et al.*, 1989). The filters were incubated with rabbit anti-adhesin serum diluted 1:50 in BLOTTO for 18 h at 4°C followed by five washes in TNT buffer, each for 5 min. The filters were then incubated at RT for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:4000 in TNT buffer–3% skimmed milk. Experiments were performed similarly with mAb F11 reactive to AP65, in which case alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad) diluted 1:4000 was used. As

negative controls, pre-bleed rabbit serum diluted 1:50 in BLOTTO or undiluted culture supernatant from the NS-1 cloned mouse myeloma cell (ATCC) was used. After incubation, the filters were washed five times with TNT buffer. Colour development was observed upon incubation in alkaline phosphatase buffer (100 mM Tris-HC1, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma). The reaction was stopped with 20 mM EDTA in phosphate-buffered saline (PBS) (Harlow and Lane, 1988).

Preparation of French-press E. coli *extracts*

Recombinant *E. coli* grown o/n at 37°C in 50 ml LB broth–ampicillin cultures were harvested, washed once, and resuspended to 1/10 of the original volume in cold 10 mM HEPES, pH 7.4, and kept at 4°C. The suspension was passed through a French pressure cell at 1260 p.s.i. in three separate cycles to lyse the bacteria. The lysate was centrifuged at 4300 × *g* for 10 min to remove intact bacteria and large debris. The concentration of protein in each lysate was ~2 mg ml^{-1} as calculated using the bicinchoninic acid (BCA) Protein Assay (Pierce). This clarified bacterial lysate was used for the ligand assay as described below.

The ligand assay

French-press bacterial lysate (1 ml) was incubated with 10^6 fixed HeLa cells for 18 h at 4°C followed by washing five times with TDSET buffer [10 mM Tris-HCl, pH 7.0, 0.2% sodium deoxycholate (DOC), 0.1% SDS, 10 mM EDTA and 1% Triton X-100]. Fixed host cells were prepared as described previously (Alderete and Garza, 1985; 1988; Arroyo *et al.*, 1992). Bacterial proteins tightly bound to fixed HeLa cells were eluted by boiling in electrophoresis sample buffer (Arroyo *et al.*, 1992) for 3 min. After SDS–PAGE, proteins were blotted onto NC for detection with adhesin antiserum or mAb F11 (Arroyo *et al.*, 1992; Lehker *et al.*, 1991).

To further show that the recombinant proteins were the parasite adhesins, a competition experiment was performed. In this case, lysates of parasites metabolically labelled for 20 h at 37°C with EXPRE^{35}S^{35}S-Protein labelling mix (specific activity 37.0 TBq $mmol^{-1}$) (Du Pont) (2.5 mCi per 50 ml of culture medium) were prepared as before (Arroyo *et al.*, 1992; Lehker *et al.*, 1991). Briefly, 2×10^7 radiolabelled trichomonads were suspended in 500 µl NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 5 mM EDTA) containing 1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma) and 100 µg ml^{-1} leupeptin (Sigma). Trichomonads were incubated in buffer with inhibitors for 10 min on ice before lysing with 1% DOC (Sigma). After 20 min on ice, the extract was vortexed for 1 min. TDSET buffer with proteinase inhibitors was then added to a final volume of 1 ml. The suspension was vortexed and then clarified by centrifugation over a sucrose cushion at 14 000 × *g* for 30 min at 4°C (Arroyo *et al.*, 1992; Lehker *et al.*, 1991).

Glutaraldehyde-stabilized HeLa cells (10^6) were first treated with different amounts of recombinant bacterial lysate or malic enzyme in TDSET buffer for 18 h at 4°C in order to ensure saturation of HeLa cell receptors before addition of ^{35}S-labelled *T. vaginalis* extract (Alderete and Garza, 1988; Arroyo *et al.*, 1992). After washing the fixed HeLa cells five times with TDSET buffer to eliminate non-specifically bound *E. coli* proteins, cells were again fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C. This was done to more accurately determine the extent of competition between recombinant proteins and adhesins. These HeLa cells were then treated with 0.2 M glycine in PBS for 1 h at RT. Finally, HeLa cells were incubated with detergent extracts containing ^{35}S-labelled trichomonad adhesins for 18 h at 4°C and then washed five times with TDSET buffer. Proteins bound to HeLa cells were eluted by boiling in electrophoresis sample buffer for 3 min (Arroyo *et al.*, 1992; Lehker *et al.*, 1991), and separated by SDS–PAGE. Gels were stained and prepared for fluorography. Cell lysates of *E. coli* containing the plasmid without any cDNA inserts were used identically as controls. Finally, the fluorographic patterns of the *T. vaginalis* adhesins were scanned using the NIH IMAGE 1.55B program in order to quantitate the extent of competition between recombinant proteins and trichomonad adhesins.

Cytoadherence assay

The assay to measure the binding of *T. vaginalis* to HeLa cells in monolayer cultures was performed using exactly the same conditions that were detailed recently (Arroyo *et al.*, 1992). For the antibody inhibition experiments with both antiserum to AP65 and mAb F11, the procedures employed were those also described previously (Arroyo *et al.*, 1992).

N-terminal amino acid sequencing of purified trichomonad AP65 adhesin

Trichomonad adhesin proteins purified by the ligand assay were subjected to SDS–PAGE. The proteins were electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). Protein bands were stained with Ponceau S. The AP65 adhesin band was excised. Microsequencing by automated Edman chemistry was performed with an Applied Biosystems Model 470-A gas-phase sequencer with an on-line 120-A PTH analyser (UTHSCSA Protein Chemistry Core Facility).

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