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Molecular characterization of a third malic enzyme-like AP65 adhesin gene of *Trichomonas vaginalis*

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Adherence to the vaginal epithelium by the sexually transmitted parasite *Trichomonas vaginalis* is mediated by four trichomonad surface proteins (AP65, AP51, AP33 and AP23). We recently showed that the 65-kDa adhesin is a member of a multigene family comprised of two similar but distinct proteins, AP65-1 and AP65-2, encoded by the genes *ap65-1* and *ap65-2*, respectively. An additional immuno-crossreactive clone, the 1.2 kb F11.1 cDNA, was isolated from a phagemid expression library and encoded a fusion protein of ~46 000 daltons (46 kDa) that bound to HeLa cell surfaces. A significant portion of the 5′ end was missing which, using the 5′-RACE method, was obtained and combined with the F11.1 clone to give a full-length cDNA. The *ap65-3* gene encoded for a protein of 567 amino acids with a molecular mass of 63.1 kDa. The gene showed 88% and 96% identity at the DNA level with *ap65-1* and *ap65-2*, respectively. Restriction mapping confirmed that the three AP65 genes are different. Southern analysis revealed that the *ap65-3* gene is present in the *T. vaginalis* genome in multiple copies. Experiments with agar clones of trichomonads showed that each gene of the multigene family is present in all parasites, and Northern analysis showed that *ap65-3* is expressed and transcriptionally regulated by iron. The *ap65-3* gene had a leader sequence and, as with *ap65-1* and *ap65-2*, showed significant homology to malic enzyme. Finally, analysis of the 3′-untranslated regions revealed that the transcript of *ap65-3* had a long poly (A) tail in comparison to *ap65-1* and *ap65-2*. Even more intriguing, sequences were found that may relate to differential degradation of select AP65 transcripts, such as the sequence motifs AUUUA for *ap65-1* mRNA and UUAUUUAU for the *ap65-2* mRNA, which were not found for *ap65-3*. © 1996 Academic Press Limited

Key words: Adhesins; cDNAs; cytoadherence; host–parasite, *Trichomonas vaginalis*; virulence.

Introduction

The sexually transmitted protozoan *Trichomonas vaginalis* is responsible for significant morbidity among women.[1–4] Adherence to the vaginal epithelium by the parasite, a property key to colonization and infection, is a highly specific event that is mediated by adhesins, designated AP65, AP51, AP33 and AP23.[5,6] These four proteins reside on the surface of *T. vaginalis*.[5–8] The association between amounts of adhesins and levels of cytoadherence has been demonstrated.[6]

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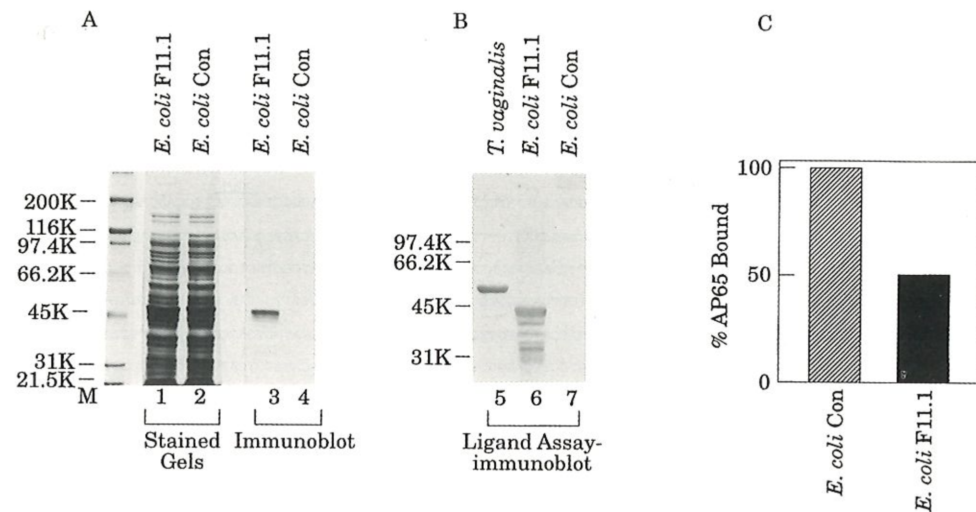

Fig. 1. Identification of recombinant F11.1 protein expressed in *E. coli* (A), which binds to HeLa cells (B) and competes with native AP65 protein (C) for binding HeLa cells. (A) Coomassie brilliant blue-stained total protein patterns and immunoblots of lysates of *E. coli* with vector alone (lanes 2 and 4) were compared with recombinant *E. coli* lysates (lanes 1 and 3). Immunoblotting was performed for visualization of recombinant proteins recognized by mAb to AP65. Molecular-size markers (Bio-Rad) are shown on the left in kDa. (B) Detection of trichomonad AP65 (lane 5) and recombinant protein (lane 6) binding to fixed HeLa cells in a ligand assay. Proteins bound to fixed HeLa cells were eluted, and after SDS-PAGE and blotting of proteins onto nitrocellulose, the native AP65 adhesin(s) and the recombinant protein were detected with mAb to AP65. No bands were seen with mAb or antiserum to AP65 when lysates of control *E. coli* harbouring plasmid alone (lane 7) were used under identical conditions. (C) Inhibition as seen by densitometric analysis of a representative experiment of binding of ^{35}S-labeled trichomonad AP65 to HeLa cells by lysates of recombinant *E. coli* F11.1 (■) but not of control *E. coli* (▨). In a ligand assay, trichomonad AP65 from ^{35}S-labelled *T. vaginalis* extracts was added to fixed HeLa cells, which were first pretreated with extracts of either recombinant F11.1 *E. coli* (F11.1) or *E. coli* containing plasmid without inserts (Con). The inhibition experiment was performed no less than three separate times and the range of inhibition was always 40% to 60%, consistent with recently published results.[10,12]

shows the native trichomonad AP65 purified from a ligand assay and immunoblotted with mAb. Proteins of *E. coli* harbouring the vector without insert used as a control in immunoblots never reacted with anti-adhesin serum or mAb (lanes 4 and 7).

Further confirmation of the functionality of the F11.1 recombinant protein was provided by a competition experiment performed with recombinant *E. coli* lysates and ^{35}S-labelled *T. vaginalis* adhesins. Pretreatment of HeLa cells with *E. coli* lysate expressing F11.1 recombinant protein decreased the amount of trichomonad ^{35}S-AP65 bound to host cells (Fig. 1C), consistent with earlier results.[12] Binding of the labeled *T. vaginalis* adhesin was not similarly inhibited when HeLa cells were first treated with equal or greater amounts of protein from control *E. coli* lysates. These results show that the F11.1 recombinant protein contains the receptor-binding epitope for HeLa cell surfaces, as does the trichomonad AP65 adhesin.

Molecular analysis of the F11.1 cDNA clone

The nucleotide sequence of the F11.1 cDNA was obtained and revealed that the insert was 1201 bp in length. The ORF encoded a protein of 363 amino acids that aligned with the C-terminal portions of AP65-1 and AP65-2. The fact that the AP65 genes were >500 bases longer than F11.1 and encoded proteins of 567 amino acids

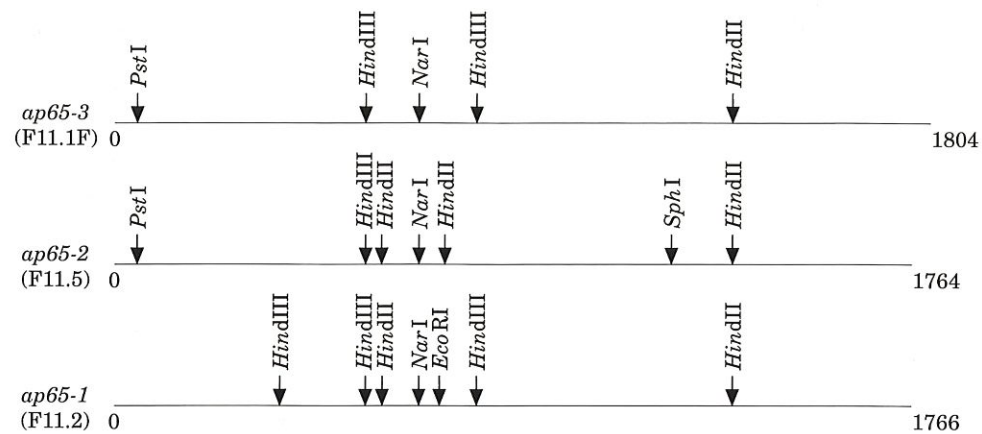

Fig. 3. Restriction analysis confirms *ap65-1* (F11.2 cDNA), *ap65-2* (F11.5) and *ap65-3* (F11.1) are different. Arrows represent restriction-endonuclease cleavage sites on the cDNAs, which were confirmed experimentally and in accordance with the sequence.

Southern analysis of restricted genomic DNA using F11.1SP as a probe generated multiple bands (Figure 4, part II), establishing that *ap65-3* is present in the *T. vaginalis* genome (lane 8). Moreover, restriction of DNA with *Eco*RI, which has no sites within F11.1F (*ap65-3*), gives multiple bands, indicating that *ap65-3* is multicopy, as seen for both *ap65-1* and *ap65-2* genes.[12]

We next analysed agar clones and subclones derived from single trichomonads in order to determine whether individual parasites contained each of the three AP65 genes. The genomic DNA of the parent population and all clones and subclones reacted identically with F11.1SP, the probe specific for *ap65-3* (Fig. 4, part II). Even more importantly, all clones and subclones reacted and gave different band patterns when the F11.2 5′-PCR and F11.5 5′-PCR products were used as probes on the same membrane (data not shown). These band patterns were identical to those found using DNA from the parental population, as recently published.[12] That each gene-specific probe hybridized and gave a distinct banding pattern indicates that the AP65 genes are non-allelic and located in different areas of the genome.

Molecular comparisons of the AP65 genes and proteins

Having demonstrated that all three AP65 genes were present in the *T. vaginalis* genome and were transcribed as shown below, it was important to carefully examine the nucleotide and amino acid sequences for any possible distinguishing characteristics. Fig. 5 presents an alignment of all three AP65 proteins. The predicted protein sequence of AP65-3 shows 89% and 96% identity to AP65-1 and AP65-2, respectively. The predicted isoelectric points were 7.29 for AP65-1, 8.12 for AP65-2, and 7.91 for AP65-3.

We reported previously that AP65-1 and AP65-2 have high homology to malic enzyme.[12] AP65-3 shows equal identity to this enzyme family. Areas that correspond to important malic enzyme domains are conserved among all three proteins (Fig. 5, overlined sequences). These include the dinucleotide binding region (sequence I), the ADP-binding $\beta\alpha\beta$ fold (sequence II), the Mn^{2+}-binding domain (sequence III), and the NADP-binding fold (sequence IV). In these domains, the AP65 proteins show almost complete identity to human malic enzyme.[12]

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                    ↓
AP65-3  MLASSVAAPVRNICRAKLPALKTGMTLLQDGDLSKGSAFTKEERDRLNLRGLLPYKVFTKDEQAARIRRQFELMPTPLLKYIFLANEREKNSQSFWRFLF  100
AP65-2  ..T...SV.........V.T................................................................................  100
AP65-1  ..T...SL.A.ELS.KV..T.............N..T...............................................................  100
                 ────I─────                                                    ───────────────II───────────────F11.1→
AP65-3  THPPTETMPVLYTPTVGEACQKWATHRQSYRGIYITPEDSGKIKDILRNYPRQDIRCIVVTDGGRILGLGDLGASGLGIPVGKLMLYTLIGQVHPDQTLP  200
AP65-2  ..............................................................A..............................N......  200
AP65-1  ....E....I....................................................A..............................D......  200
                                                                        ─────────────────III────────────────
AP65-3  VQLDMGTDRKEILADPLYHGWRHPRIRGPEHTKFVAEFVDAVKEVFGETCLVQFEDFEMETAFKLLDHFRWRCNCFNDDIEGTAAVAAATLASATHMEGV  300
AP65-2  ...............................................................T....................................  300
AP65-1  .........................V..A..L...T...........D....................................................  300
                  ───────IV─────────
AP65-3  PKLKNQKIIFIGAGSAATGIANLIVDMAVSRGGISRKDAERNIIMFDHKGMVHADRKDLYDFNKPYMHDMEVYGSVLEGVKKFKATCVIGVSGVPGLITK  400
AP65-2  ..................................TKEQ.YK.............G.............N.................S.............  400
AP65-1  .................I.........T......TKEQ.YK.............G.......................A.....................  400

AP65-3  EIVQATCANCERPVIMPLSNPTVKAEAKPHDVYQWSNGKALCATGSPFPVETVNGKKTITAQANNSWIFPAVGYALVTTRARHCPGKVFEVAAESLASLV  500
AP65-2  .....A..............................................................................................  500
AP65-1  ......LK.A............P....T.....L...............A.QV..R.V.....................K.....A....I.........  500

AP65-3  KKEDHDMGNLLPPLDKIREYSFGIALDVAKYLIKNELATALPPKGTELKDWLKAQLFDPQAEYEQLY                                  567
AP65-2  ..............N...D.....................V..........................                                  567
AP65-1  ......Q.......N...D......Y..S....D......V.....S..........E.S.D..P..                                  567
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Fig. 5. Alignment of the predicted amino acid sequences of the three AP65 adhesins. The arrow denotes the putative cleavage site of the leader peptides (to the left of the arrow). The beginning of the F11.1 recombinant protein is indicated. The overlined segments correspond to conserved malic enzyme domains, including the dinucleotide-binding region (I), the ADP-binding $\beta\alpha\beta$ fold (II), the Mn^{2+}-binding domain (III), and the NADP-binding fold (IV).

A 5′ UTRs aligned with the *T. vaginalis* consensus promoter sequence

Gene (clone)	Sequence
ap65-3 (F11.1-C38)	T T T T A G A T T A A A G A T G
ap65-2 (F11.5-C2)	T T T C A G A T T A A A G A T G
ap65-1 (F11.2-C1)	T T T T T G A T T A A A G A T G
Consensus	T C A T/C T T/A T/C T C A T T A

B Comparison of 3′ UTRs

Gene (clone)	Sequence
ap65-3 (F11.1)	TAAGCAGTTTTTAAAACTCTTTCAATTGTCTTTG(55 A)
ap65-2 (F11.5)	TAAATCTATTAAAGTTTCTGGTTGATTATTTATG(15 A)
ap65-1 (F11.2)	TAAGCGCGATTTTTAAACACTTAGCTTTCTTAATTTA(15 A)
Consensus	TTATTTATT

Fig. 7. Comparative analysis of the 5′ and 3′ untranslated regions (UTRs) of the AP65 adhesin genes. (A) Representative 5′ UTRs of each gene aligned with the consensus sequence for *T. vaginalis* promoters.[25] Shaded areas indicate bases that agree with the consensus sequence. The translational start site (ATG) is underlined. The box denotes gene-specific bases at the -9 and -10 positions. (B) The 3′ UTR of each AP65 gene from the underlined stop codon (TAA) to the poly-A tail indicated parentheses. The mRNA sequence pattern AUUUA, which is found in AU-rich elements and linked with mRNA instability,[22] is indicated (double underlined). The sequence of the recently reported destabilizing element TTATTTATT[20] is shown for *ap65-2* (highlighted area) and is presented underneath as a consensus 3′ UTR. Shaded nucleotides agree with the consensus sequence.

the 10 bp area that overlaps the consensus sequence; all have gene-specific bases at the -9 and -10 positions relative to the start site.

Interestingly, the AP65 genes show little or no homology in the 3′-UTRs (Fig. 7B). The transcripts for *ap65-1* and *ap65-2* have short 15 bp poly (A) tails relative to that of *ap65-3*, which has a 55 nucleotide poly (A) tail. Also, in contrast to *ap65-3*, the 3′-UTRs for *ap65-1* and *ap65-2* contain a sequence motif (ATTTA in the cDNA and AUUUA in the mRNA) found in AU-rich elements (AREs), which have been shown to confer instability on mRNAs.[22] Furthermore, the destabilizing sequence element (UUAUUUAU)[20] was found in the 3′-UTR for *ap65-2*, showing another difference between the mRNAs of these genes.

Discussion

This record presents evidence showing the existence of *ap65-3*, a third gene that encodes the AP65-3 adhesin. The F11.1 cDNA was isolated from an expression library that encoded epitopes immuno-crossreactive with antiserum against *T. vaginalis* AP65.[10] Restriction mapping (Fig. 3) and Southern analysis using non-cutting enzymes (Fig. 4) confirmed that the three AP65 genes are non-identical and that *ap65-3* is also multicopy in the *T. vaginalis* genome. Furthermore, examination of cloned trichomonads showed that the AP65 genes are non-allelic. That we have discovered three members or isoforms of the AP65 multigene family, that each of those genes is present in multiple copies, and that all three

Furthermore, the three AP65 genes were found to be regulated by iron (Fig. 6).[8,12,30] If, in fact, one of the AP65 proteins is the *T. vaginalis* malic enzyme,[26] these findings show that this and possibly other hydrogenosomal enzymes are up-regulated by iron. A recent study shows that the activities of *T. vaginalis* metabolic enzymes vary depending on culture conditions.[31] Thus, studies cannot ignore the role of certain factors in regulating expression of *T. vaginalis* enzymes and, therefore, in detection of trichomonad enzymatic pathways.

Contact with VECs or HeLa cells induces trichomonads to synthesize greater amounts of all four adhesins,[9] and we hypothesized that utilization of internal iron pools led to adhesin synthesis.[9] Although speculative, it may be necessary to consider that adhesin synthesis is regulated by the rate of transcript turnover. Other recent studies have proposed that the protozoan parasite Plasmodium possibly regulates expression of the *var* multigene family at the post-transcriptional level by degradation of select mRNAs.[32] It is not inconceivable that the AP65 genes are continuously transcribed, in order to provide basal levels of adhesins, as shown,[8] but that the majority of the transcripts may be rapidly degraded. There are reports, for example, that show mRNA turnover involves specific sequences and regions that contribute to the instability of transcripts.[19,20,22,33] One sequence element involved in RNA destabilization is the AU-rich element (ARE).[19,20] It may be significant that the 3′-UTRs of *ap65-1* and *ap65-2* each contain the AUUUA sequence motif often found in AREs and, in addition, both transcripts have short poly(A) tails (Fig. 7B). Furthermore, a recent report shows that the octamer UUAUUUAU, found in *ap65-2*, effectively mediated deadenylation and rapid decay of mRNA.[20] In contrast *ap65-3* does not contain these motifs and has a long poly(A) tail. Since the ARE may promote deadenylation and subsequent mRNA destabilization,[21,22] differential degradation of the transcripts might explain why *ap65-1* (F11.2) and *ap65-2* (F11.5) are expressed in small amounts in *E. coli*. In contrast, *ap65-3* (F11.1) has higher levels of expression. The signalling event described earlier may lead to a decrease in mRNA turnover, especially of the *ap65-1* and *ap65-2* transcripts, providing for a mechanism of rapid expression of the individual AP65 proteins. Further experiments comparing the levels of transcript for each gene before and after interaction with host cells will be needed to elucidate the relationship between the signalling event, turnover of individual AP65 transcripts, and the possible role of the UTRs.

Interestingly, there is precedence for the post-transcriptional regulation of ligands. The gene expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), an enzyme reported to have a number of functions, including adherence,[15,16,34–36] has been shown to be regulated at the post-transcriptional level in rat tissue by adjusting mRNA abundance.[38] Although the mechanism of regulation was not known, different levels of GAPDH gene expression correlated with differential polydenylation. In group A Streptococci, the intracellular form of GAPDH functions as a metabolic enzyme, whereas the surface-located form functions as a plasmin receptor.[15,16] Since AP65 and GAPDH may both be metabolic enzymes also recruited for use on the cell surface as adhesins, the finding that they may be similarly regulated by mRNA turnover is potentially significant.

Materials and methods

Microorganisms and culture conditions. *Trichomonas vaginalis* isolate T016N was a fresh clinical isolate which expressed high amounts of adhesins under iron-replete growth

nitrocellulose (NC) for immunoblotting.[48] Immunoblotting was performed as previously described[12] using mAb F11 reactive to AP65 or with rabbit anti-AP65 serum. As negative controls, a mAb of the same isotype but to a non-adhesin protein or prebleed rabbit serum were used.

Preparation of French press* E. coli *extracts. Recombinant *E. coli* lysates were prepared by French press as described before.[12] This clarified bacterial lysate was used for the ligand assay as described below.

The ligand assay. A ligand assay, described previously[5,6,12] was used to analyse the recombinant proteins. Briefly, French-press bacterial lysate (1 ml) was incubated with 10^6 fixed HeLa cells for 18 h at 4°C before washing to remove non-specific binding. Bacterial proteins bound tightly to fixed HeLa cells were eluted by boiling in electrophoresis dissolving buffer[12,45] and subjected to SDS-PAGE and subsequent immunoblotting with anti-AP65 antibodies or controls.

To further show that the recombinant protein was a functional adhesin, a competition experiment was performed numerous times as recently detailed.[12] In short, glutaraldehyde-stabilized HeLa cells (10^6) that were first treated with 500 μL of recombinant bacterial lysate were incubated with lysates of parasites metabolically labelled with EXPRE^{35}S^{35}S-Protein labelling mix[5,6] and then washed. Proteins bound to HeLa cells were eluted by boiling in electrophoresis dissolving buffer for 3 min,[6,8] 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. The X-ray films representing patterns of the *T. vaginalis* adhesins bound to HeLa cells after SDS-PAGE-fluorography were scanned using the NIH *Image* 1.55b program in order to quantitate the extent of competition between radiolabelled recombinant protein and native trichomonad adhesins. Results of this competition experiment were reproducible and consistent with those shown recently[10,12] for other AP65 recombinant adhesins.

***Agar cloning of* T. vaginalis.** Agar clones and subclones derived from *T. vaginalis* isolate T016N were isolated by using a modification of a previously-published procedure.[46,49] Briefly, 35.5 ml of TYM containing 0.65% noble agar (Difco Laboratories, Detroit, MI) was autoclaved and incubated in a 60°C water bath for 20 to 30 min. The medium was then transferred to a 37°C water bath and 1 ml of stock penicillin/streptomycin solution (100 μg/ml final concentration), 4 ml of HIHS, and 1 ml of inoculum containing 20 to 40 organisms were added immediately upon temperature equilibration. The contents were mixed briefly and poured into sterile glass petri dishes (100 by 15 mm) with Whatman filter paper taped to the inside of the glass cover. The plates were placed in a Brewer jar with BBL GasPak Plus (Becton Dickinson and Co., Cockeysville, Md.) and incubated at 37°C for 7 to 8 days. Visible colonies were then inoculated into sterile 0.1% TYM-agar and transferred to TYM-10% HIHS for cultivation by routine methods.

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