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Only two of the *Trichomonas vaginalis* triplet AP51 adhesins are regulated by iron

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The sexually transmitted parasite *Trichomonas vaginalis* cytoadheres to the vaginal epithelium, and four candidate trichomonad adhesins have been identified. One such protein, termed AP51, was characterized further. To do this, we studied a 1 kb cDNA clone (AP51.2) isolated from a phagemid expression library, which encoded a fusion protein of ~38 kDa that was immuno-crossreactive with anti-AP51 serum and retained functional adhesive properties. We performed 5′-PCR amplification to recover the missing 5′ end in order to provide the complete cDNA sequence for the gene encoded by AP51.2 (*ap51-2*). Other PCR products revealed almost complete sequences for two additional *ap51* genes, making AP51 a member of a multigene family of at least three distinct proteins and genes. The *ap51-1* and *ap51-3* genes each encoded for 407 amino acids while *ap51-2* encoded 408 amino acids, and not unexpectedly, these genes had a high percent identity at the DNA and amino acid levels. Mapping confirmed the sequence distinctions and uniqueness of the three *ap51* genes. Southern analysis using gene-specific probes revealed the single copy nature of each of the *ap51* genes, all of which were present among the numerous agar clones of single trichomonads of the isolates tested. Importantly, Northern analysis showed transcriptional regulation by iron of only the *ap51-1* and *ap51-3* genes but not *ap51-2*, perhaps indicating the presence of two bona fide isoforms of the *ap51* genes. The 3′-untranslated region of *ap51-3* had a short poly (A) tail as well as the sequence motif AUUUA, which may relate to differential degradation of *ap51-3* transcripts, in comparison to *ap51-1* and *ap51-2*. Finally, the *ap51* genes had partial homology to the β-subunit of succinyl-CoA synthetase, reinforcing the idea that molecular mimicry may play a role in host parasitism by *T. vaginalis*.

Key words: Adhesins, cDNAs, cytoadherence, genes, host-parasite, *Trichomonas vaginalis*.

Introduction

Trichomonas vaginalis is the number one non-viral sexually transmitted agent worldwide [1]. Infection with this parasite has recently received increased attention. This sexually transmitted parasite causes unpredictable symptomatology [2], which can range from a relatively asymptomatic carrier state to one of severe foul-smelling discharge, irritation and discomfort accompanied with inflammation and tissue cytopathology. It is now appreciated that patients

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with trichomonosis (vaginitis) are at higher risk for HIV seroconversion [3], for adverse outcomes during pregnancy [4], and for cervical cancer [5].

Pathogenesis likely begins with specific colonization of the vaginal epithelium [6]. Cytoadherence appears to be mediated by four distinct candidate trichomonad surface proteins that have been termed AP65, AP51, AP33 and AP23 [7, 8]. Signaling for adaptive responses occurs upon binding by trichomonads to host cells [9]. For example, upon contact with vaginal epithelial cells (VECs), a rapid increase in synthesis of the four adhesin proteins was demonstrated [9]. In addition, an equally rapid and dramatic transformation in morphology, the rate and extent of which was directly related to the amount of surface-expressed adhesins, was detected [9]. The level of cytoadherence to VECs was equally related to the amount of the four proteins on the parasite surfaces [8, 10]. Importantly, when HeLa cells were used as the host cells under similar experimental conditions, although there was an increase in adhesin synthesis, no signaling of the parasite toward morphological change was evident, showing specificity and *in vivo* significance in the signaling pathways [9]. It is equally significant that upregulation of expression of the genes encoding the four proteins appears to be under the control of iron [10]. It seem clear, therefore, that the goal of understanding the pathogenesis of trichomonosis requires a priori characterization at the molecular level of these candidate proteins involved in cytoadherence.

Recently, we found that one of the adhesins, termed AP65, was a member of a multigene family, with each gene present within individual parasites, and each of the three *ap65* genes existed in the genome of trichomonads in multiple copies [11–13]. Northerns revealed regulation at the transcriptional level by iron of the three *ap65* genes [11–13]. Of particular significance was the strong homology of the surface AP65 trichomonad adhesin with a metabolic enzyme at the nucleotide and amino acid sequence levels [11], indicative of enzymes with alternative functions on surfaces of pathogenic organisms [14–18] and suggesting gene sharing and molecular mimicry [19, 20].

This paper describes AP51, the second of four candidate adhesins and corresponding genes [8, 11]. We show that iron upregulated expression of only two of the three *ap51* multigene family members, which is in part consistent with our earlier results [10, 11]. Similar to the *ap65* genes, there is homology between the ap51 genes and an enzyme subunit, and one transcript also possessed in its 3′ UTR a destabilizing element reported to regulate rates of mRNA turnover [21–23]. These data now direct our attention to the possibility of isoforms for the members of the multigene family, one possibly representing a constitutively expressed enzyme and the other being environmentally regulated adhesins. The complex nature of the pathogenesis of trichomonosis is evidenced by the highly evolved and sophisticated strategy for host parasitism by this sexually transmitted protozoan.

Results

Properties of the natural and recombinant trichomonad AP51 proteins

The four trichomonad surface proteins previously identified as the putative adhesins are shown in Figure 1 (Ia). Stained gels of proteins used for standard size estimations (lane 1) were used to analyse the trichomonad proteins identified by the ligand assay [7, 8] (lane 2) (Materials and methods) after electrophoresis. Figure 1 (Ib) (lane 3, bracket) illustrates that rabbit antiserum generated to the protein termed AP51 eluted from acrylamide gels (lane 2, arrow) readily reacted by immunoblot with the AP51 protein from a duplicate gel as that of lane 2. The multiple bands of less intensity may be due to the established susceptibility of these proteins to degradation by the cysteine proteinases released upon lysis of trichomonads. No immunoreactivity was evident with control, prebleed rabbit serum (shown below).

Immunoscreening of the cDNA library originally yielded only one clone (AP51.2) that was immunoreactive with anti-AP51 serum [11]. Analysis of the recombinant protein by immunoblot after SDS-PAGE of *E. coli* detergent lysate demonstrated a readily visible and abundant fusion protein band with M_r of $\sim$38 kDa in stained gels [Figure 1 (Ib), lane 7]. No similar intense stained band was present in the control of *E. coli* lysate harboring the vector alone (lane 4). This recombinant AP51 protein was presumed truncated when the M_r was compared to that of the natural AP51 protein (lanes 2 and 3). The recombinant protein bound to HeLa cells, as evidenced by using a French Press extract

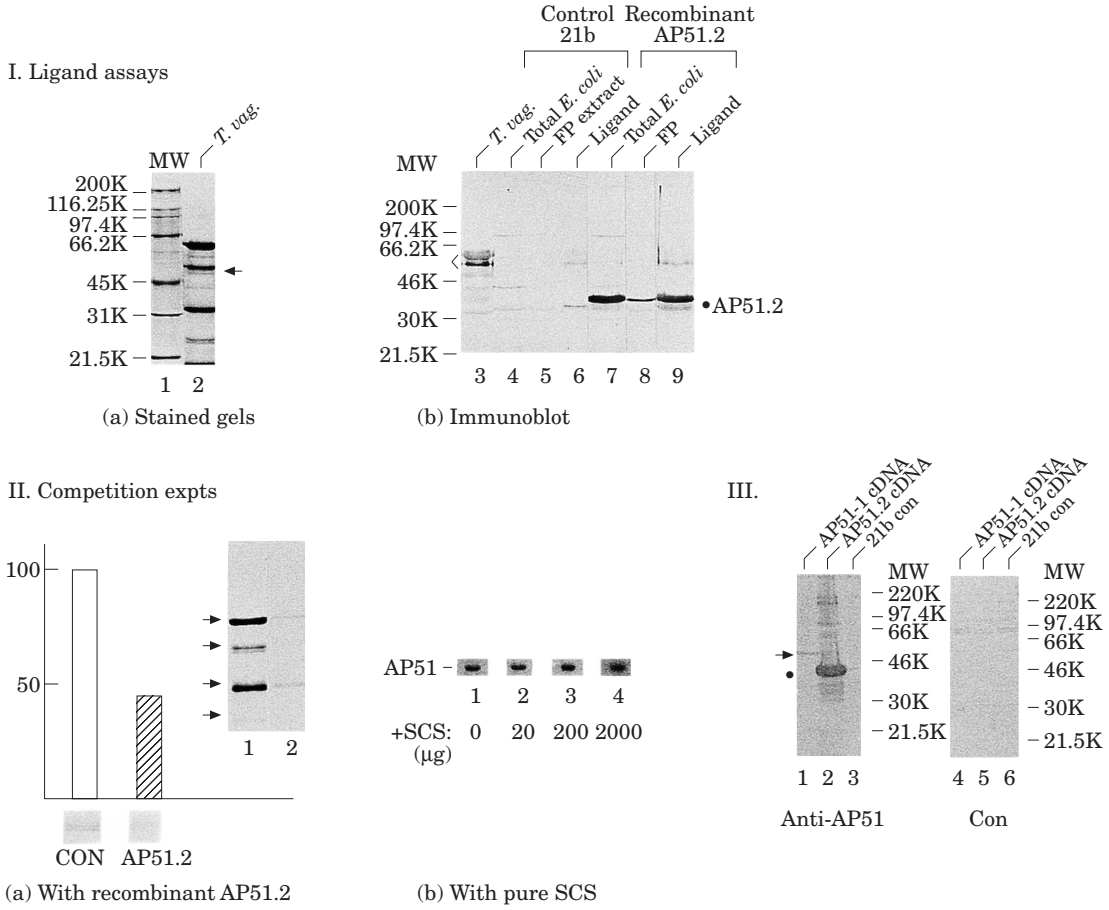

Figure 1. The AP51 proteins express biofunctionality and have receptor-binding epitopes unique from β-succinyl-coenzyme A synthetase-like sequences. (I) The four trichomonad proteins seen in Coomassie brilliant blue-stained acrylamide gels [part (a), lane 2] were identified by the ligand assay (Materials and methods). One protein was termed AP 51 (arrow) based on its electrophoretic mobility in comparison with stained molecular size markers (lane 1). Part (b) shows the immunocrossreactivity of the natural AP51 (lane 3) from nitrocellulose blots of proteins obtained in lane 2 probed with anti-AP51 serum characterized previously [8, 11]. Lanes 7 through 9 show the reaction of a smaller, truncated recombinant protein encoded by cDNA AP51.2 isolated from an expression library [11]. Recombinant protein was detected in total *E. coli* lysates (lane 7), a French pressure cell extract (lane 8), and protein eluted from fixed host cells in the ligand assay (ligand, lane 9). No crossreactive proteins were detected in lysates of control *E. coli* with vector alone (lanes 4 and 5) and from the ligand assay performed with control *E. coli* (lane 6). (II) Recombinant protein [part (a)] but not commercially available pure SCS enzyme containing both α and β SCS subunits, inhibits binding of ^{35}S-labeled trichomonad AP51. The fluorogram gel pattern in parts (a) and (b) shows the adhesin proteins after the ligand assay and electrophoresis. Radiolabeled bands from fluorograms of the competition experiment are shown as individual bands below the bar graphs in part (a) and in part (b) (lanes 1–4). Control refers to a competition experiment performed with *E. coli* containing vector without insert [part (a)] or is indicated by the absence of added SCS [part (b), lane 1]. In the ligand assay, ^{35}S-labeled AP51 in *T. vaginalis* extracts was added to fixed HeLa cells, which were first pretreated with either recombinant *E. coli* expressing the truncated AP51 protein [part (a)] or pure SCS [part (b)] at the amounts indicated (lanes 2–4). Insert shows proteins after ligand assay and electrophoresis following a competition experiment in which HeLa cells were first pretreated with an extract of unlabeled *T. vaginalis* isolate T016. Note the decrease in binding of all four trichomonad proteins, as expected because of the coordinated synthesis of adhesins (lane 2) compared to control untreated cells handled identically (lane 1). (III) Immunodetection with anti-AP51 serum after electrophoresing and transfer onto nitrocellulose of the full-length AP51 recombinant protein corresponding to *ap51-3*, called AP51-1 cDNA (lane 1), derived from combined 5′ and 3 RACE products (Results and Materials and methods). This was compared with the strong reactivity always obtained with the truncated AP51.2 cDNA product (lane 2). No bands were ever detected with antiserum of control *E. coli* extracts (lane 3). Similar absence of band reactivities were detected in all samples probed with control (Con), prebleed rabbit serum (lanes 4–6).

Figure 2. See caption opposite.

(lane 8) of *E. coli* expressing AP51.2 cDNA in a ligand assay (lane 9). Not unexpectedly, detergent lysates and French Press extracts of control *E. coli* in the ligand assay gave no reactivity in immunoblots handled identically (lanes 5 and 6).

Finally, part IIa shows that only extract of *E. coli* with insert (labeled AP51.2) but not control *E. coli* (labeled con) produced a decrease in the amount of ^{35}S-labeled trichomonad AP51 bound to host cells. Below the bar graph is a representative fluorogram showing relative in-

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ap51-1  ACTTCGATGATTCCGCTCACTACCGCCAGAAGCAGATCTTCGGTTACCGCGATCTCAAGCAAGTCAACCCATTCGAAATCCGCGCTGAGAAGTATGGTCT 800
ap51-2  ACTTCGATGACTCCGCTCACTACCGCCAGAAGCAGATCTTCGGCTATCGTGACCTCAAGCAAGTCAACCCATTCGAAATCCGCGCTGAGAAGTACGGTCT 800
ap51-3  ACTTCGATGACTCCGCTCACTACCGCCAGAAGCAGATCTTCGGCTATCGTGACCTCAAGCAAGTCAACCCATTCGAAATCCGCGCTGAGAAGTACGGTCT 800
        ********** ********************************** ** ** ** ********************************************** *****

ap51-1  TAACTACGTCCCACTCGATGGTAACGTTGCTTGCCTCGTTAACGGTGCTGGCCTTGCTATGGCTACAATGGATGTCATCAAACTTGCTGGTGGCGATCCA 900
ap51-2  TAACTACGTCCCACTCGATGGTAACGTCGCTTGCCTCGTTAACGGTGCTGGCCTTGCTATGGCTACAATGGATGTCATCCAACTTGCCGGTGGTGATCCA 900
ap51-3  TAACTACGTCCCACTCGATGGTAACGTCGCTTGCCTCGTTAACGGTGCTGGCCTTGCTATGGCTACAATGGATGTCATCAAACTTGCCGGTGGTGATCCA 900
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ap51-1  GCTAACTTCCTCGATCTCGGTGGTGTGCTGCTTCTGAGGCTGCTGTTACAGAGGGCTTCACAATCATCTCACAACAGTCCCACGTCAAGGCTATCCTTGTCA 1000
ap51-2  GCTAACTTCCTCGATCTCGGTGGTGGTGCTTCTGAAGCTGCCGTTACAGAGGGCTTCACAATCATCTCATCTCAGCCACACGTAAAGGCTATCCTCGTCA 1000
ap51-3  GCTAACTTCCTCGATCTCGGTGGTGCTGCTTCTGAGGCTGCCGTTACAGAGGGCTTCACAATCATCTCATCTCAGTCACACGTAAAGGCTATCCTCGTCA 1000
        ********************** ********** ********************************** .*** * ***** ********** ****

ap51-1  ACATCTTCGGTGGTATCGTTCGTTGCGATATGGTCGCTGCTGGTGTCATCGCTGCTTTCAAGAAGGTCGGTCTCAAGGTCCCACTCGTTGTCCGTCTCGA 1100
ap51-2  ACATCTTCGGTGGTATCGTCCGCTGCGACATGGTCGCTGCTGGTGTCATCGCCGCCTTCAAGAAGGTTGGCCTCAAGGTCCCACTCGTTGTCCGCCTCGA 1100
ap51-3  ACATCTTCGGTGGTATCGTCCGCTGCGACATGGTCCGTGCTGGTGTCATCGCCGCCTTCAAGAAGGTTGGCCTCAAGGTCCCACTCGTTGTCCGTCTCGA 1100
        ********************** ** ****** ******  ********************** ** ********** ** ********************** *****

ap51-1  GGGTACAAACGTCGAGGCAGGCAAGAAGCTCATCCGCGAATCTGGTCTCCCAATCATCTCAGCCGATAACCTTACAGATGCAGGTGAGAAGGCTGTTAAG 1200
ap51-2  GGGTACAAACGTCGATGCCGGTAAGAAGCTCATCCGCGAATCCGGCCTCCCAATCATCCCAGCTGACAACCTTACAGACGCTGGTATCAAGGCTGTCAAG 1200
ap51-3  GGGTACAAACGTCGATGCTGGTAAGAAGCTCCTCCGCGAATCCGGTCTCCCAATCATCCCAGCTGACAACCTTACAGACGCTGGTATCAAGGCTGTCAAG 1200
        ********************.** ** ********** ********** ** ********************** **** ** ********** **.***.. ******** ***

ap51-1  GCTGCCAAGGGCGAAAAGTTC<b>TAA</b>AAATCATCTCATAAATTTTTCTCTAGCCAGAAAAAAAAAAAAAAAAAAAAAAAAAAAA                 1279
ap51-2  GCTGCTAACGGTGAGAAGCTCATC<b>TAA</b>AGAGGTTTGCAAATTAAAATTCTAAAGTTATCAACATCTGATTCAAAAAAAAAAAAAAAAAAAAAAAAA 1300
ap51-3  GCTGCTAACGGTGAAAAGCTC<b>TAA</b>AAGAATTTTGCTC<b>ATTTA</b>ATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                 1257
        ***** ** ** **.*** **.. ....  . .  * .......

ap51-1                                                                                                      1279
ap51-2  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                                      1329
ap51-3                                                                                                      1257
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Figure 2. Nucleotide sequence of the *ap51* genes. Primers used for the Marathon cDNA amplification kit to generate both 5′ and 3′ RACE products for each gene as well as the full-length *ap51-3* clone (Materials and methods) are those illustrated in Table 1. The translational start codon (ATG) and stop codon (TAA) are noted in bold. The boxed sequences were used to generate gene-specific oligonucleotides for use as probes. The 3′ UTR illustrates the size of the poly A tail for each gene. In addition *ap51-3* has the ATTTA destabilizing sequence 13 nucleotides from the stop codon. The shaded nucleotides at the 5′ end (shaded area) were derived from the known sequence of genomic DNA identical to *ap51-1*.

Table 1. Identity of nucleotide primers and PCR conditions used to generate the *ap51* gene sequences.

Gene	Internal primers	PCR conditions
ap51-1	51CO5E (antisense)	25 cycles: 94°C, 30 s; 68°C, 3 min
	AP51P1 (antisense)	25 cycles: 94°C, 30 s; 68°C, 3 min
ap51-2	AP51P1 (antisense)	25 cycles: 94°C, 30 s; 68°C, 3 min
ap51-3	AP51P3 (antisense)	25 cycles: 94°C, 30 s; 68°C, 3 min
	51T (sense)	25 cycles: 94°C, 30 s; 68°C, 5 min

tensities of bound ^{35}S-AP51. The insert further shows, as previous results had already demonstrated [11–13] that incubation of HeLa cells first with unlabeled trichomonal extract followed by addition of the same amount of a detergent preparation of ^{35}S-labeled *T. vaginalis* resulted in decreased binding of all of the adhesins (lane 2). No similar decrease in binding of trichomonad proteins was ever detected with the control *E. coli* (data not shown). This evidence strongly supports the notion that recombinant AP51.2 protein possesses function equivalent to the natural trichomonad protein.

Isolation and molecular analysis of the full-length sequences

Sequencing the AP51.2 cDNA revealed the absence of a complete 5′ end, consistent with the size difference evident when compared to the immunoreactive parent AP51. We performed the Marathon™ in an attempt to isolate products that would allow us to complete the *ap51* gene sequence. Numerous independent clones were generated, and these clones had sequences different to that overlapping the AP51.2 cDNA sequence, which contained the primer sequence (Materials and methods and Table 1). Once we had obtained the adaptor-ligand double-stranded cDNA library, we performed PCR amplification to complete the gene sequences.

The sequence corresponding to the gene represented by the AP51.2 cDNA is shown in Figure 2 (labeled gene *ap51-2*). It is noteworthy that despite the isolation of many PCR products generated to obtain the 5′ ends, we were unable, for unknown reasons, to generate the remaining short 5′ ends to complete the *ap51* genes. Homology matching revealed that *ap51-2* was identical to a known genomic sequence [24] (discussed below), and our sequence lacked 8 nucleotides for the complete gene. Thus, the AP51.2 cDNA represented a gene of 1227 bps. The total size of the complete transcript, including the complete 5′ end and the 3′ untranslated region (UTR) (described below), was 1329 bps.

Numerous other PCR-derived 5′ clones were sequenced, yielding two new AP51 genes. The 3′ amplification was performed using primers (Table 1) that were within the 5′ region of the other putative *ap51* genes. We then isolated and sequenced numerous 3′ end PCR clones to obtain the complete sequences for *ap51-1* and *ap51-3*, and the complete alignment and comparison with *ap51-2* is presented (Fig. 2). As with *ap51-2* and based on known genomic sequence [24], both *ap51-1* and *ap51-3* lacked 8 nucleotides and 4 nucleotides, respectively, at the 5′ end. The ORF for both *ap51-1* and *ap51-3* was 1224 bps. The total size of the cDNA clone (minus the incomplete 5′ ends) for *ap51-1* was 1271 bps and for *ap51-3* cDNA clone was 1253 bps. The stop codons for the genes were at positions 1222 for *ap51-1* and *ap51-3* and 1225 for *ap51-2*.

We next attempted to isolate at least one full-length cDNA for a member of the three genes. PCR was performed using primers from both ends of the gene sequence. We successfully isolated a full-length clone equivalent to *ap51-3* identified as AP51-1 cDNA (Fig. 1, part III, lane 1). We detected a fusion protein of the expected size on nitrocellulose blots of electrophoresed extracts of recombinant, but not control *E. coli*, with anti-AP51 serum (lane 1 versus lane 3). This protein was not over-expressed when compared to the original AP51.2 clone in the same vector (lane 2). It is noteworthy that recombinant *E. coli* harboring either the original AP51.2 clone or the full-length *ap51-3* clone grew to similar optical densities, suggesting no overt toxicity by the expressed recombinant proteins. In these comparative experiments, identical amounts of total *E. coli* proteins were added for immunoblotting, as evidence by equal intensity of

stained gel bands after electrophoresis (data not shown). Prebleed, normal rabbit serum did not react with any recombinant (lanes 4 and 5) or control (lane 6) *E. coli* proteins. The finding of a 3′-UTR destabilizing element [21, 22] in two recently described *ap65* genes [13] prompted us to evaluate as much of the 3′ noncoding sequences for the *ap51* genes that we could isolate.

Figure 2 presents the 3′-UTR sequence confirmed from several PCR clones representing each gene, each of which was derived from 3′ Marathon amplification. The representative clones for each gene had a poly (A) tail of 30 A's for *ap51-1* and a long stretch of 58 A's for *ap51-2* in contrast to *ap51-3*, which had a short tail of 17 A's. The destabilizing sequence ATTTA (AUUUA in the mRNA), which is found in AU-rich elements (AREs) and has been shown to confer instability to mRNAs [21–23], was found after the stop codon in the 3′-UTR of *ap51-3* (13 nucleotides after the stop codon). In summary, the finding of the destabilizing element for *ap51-3* but not for *ap51-1* and *ap51-2*, and the varying amounts of recombinant protein produced in *E. coli* (Fig. 1), for unknown reasons, serve to further differentiate the transcripts and proteins for AP51.

AP51 represents a family of three single copy genes present in all trichomonads

Southern analysis produced the expected hybridization by the AP51.2 clone of all cDNAs that represented the three genes [Fig. 3, part (Ia)]. As a control, the ^{32}P-labeled AP51.2 clone did not hybridize with cDNAs representative of either the *ap65* and *ap33* adhesin genes [10, 13]. As can also be seen, Figure 3 shows that each gene-specific oligonucleotide (Fig. 2, boxed region) reacted with only the cDNA from which it was derived [part I, (b)–(d)].

Each gene probe was hybridized to the genomic DNA from numerous *T. vaginalis* isolates. The DNA was restricted with endonucleases unable to cut within each gene. Part II shows the comparative hybridization patterns using the AP51.2 cDNA versus each respective gene-specific probe. One band was seen for each gene, indicating the existence of only a single copy. Not unexpectedly, the pattern using AP51.2 (part IIa) as the probe was a composite of individual blots when the oligonucleotide probes were used singly on the same blot (part II, b through d). We then demonstrated the existence of the three genes in the genome of individual agar-cloned trichomonads. Figure 4 presents the Southern analysis for the parental isolate and three representative agar-subcloned parasites, showing conclusively the non-allelic nature for the genes.

Only ap51-1 and ap51-3, but not ap51-2, show transcriptional regulation by iron

Finally, we tested for the regulation of gene expression by iron for each of the three *ap51* genes using antisense oligonucleotides of the gene-specific probes used for Southerns above (Fig. 3). This was necessary because of the established fact that iron coordinately regulated the synthesis of the adhesins, as evidenced by amounts of mRNA [11–13] and the corresponding adhesin proteins [10]. Autoradiograms from Northern analysis of mRNA (Fig. 5a and 5c) showed that the probes specific for the *ap51-1* and *ap51-3* genes hybridized to a transcript of expected size ($\sim$1.2 kb) from mRNA isolated from high-iron trichomonads. Longer exposure of X-ray films was required to reveal the presence of detectable transcript from Northerns performed with mRNA of low-iron-growth parasites when amounts equal to that used above for high-iron organisms were used. The fact that similar results were obtained for *ap51-1* and *ap51-3* by Northerns of agar-subcloned organisms (not shown) established that the two genes were each transcribed and regulated similarly by iron. Surprisingly, detection of transcript bands of equal intensity by a probe specific to *ap51-2* occurred with mRNA from both high- and low-iron-grown organisms. As controls, the antisense oligonucleotide probes for Northerns were also employed in Southern analysis as above (Fig. 3), and identical results were obtained in the same Southern blots, showing the specificity and reactivity of the probes with the individual *ap51* genes. These Southern and Northern analyses are in agreement with the detection of surface AP51 for all isolates that have been examined in our laboratory [6–13], some of which are included in this report. These data show that the *ap51* genes are distinguishable in terms of environmental regulation of expression and further point toward the presence of two bona fide isoforms of these genes.

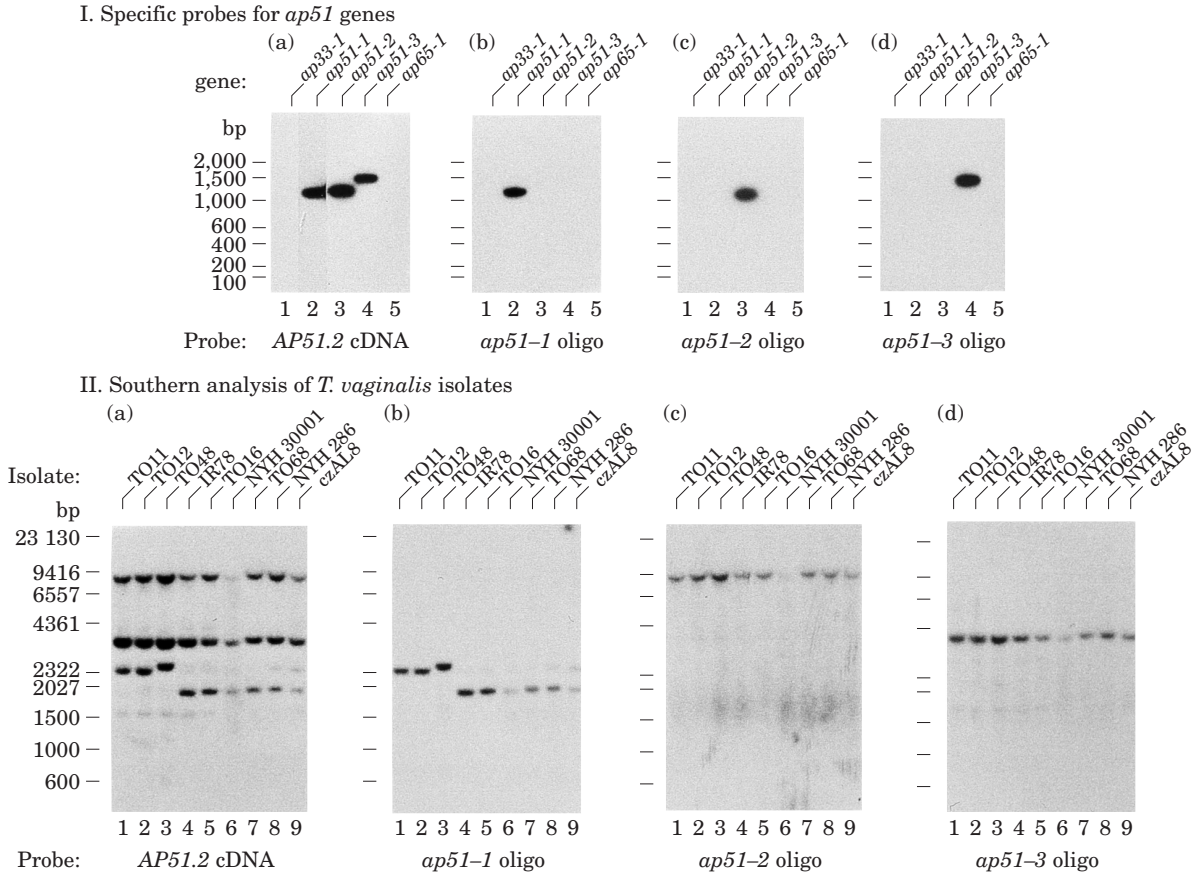

Figure 3. AP51 gene-specific probes hybridize uniquely, and each shows that the *ap51* genes are single copy. (I) The individual probes (labeled *ap51-1* oligo for *ap51-1* gene-specific, *ap51-2* oligo, and *ap51-3* oligo) hybridize only to cDNAs from which they were derived [parts (b) through (d)] in contrast to the truncated cDNA designated AP51.2, which hybridizes to all three cDNAs representing the three *ap51* genes. The radiolabeled *ap51-1* oligo hybridized only to the *ap51-1* cDNA isolated from pCRII vector (lane 2). Likewise, the other two *ap51-2* and *-3* oligos hybridized only with their respective cDNAs. No hybridization was evident with cDNAs of *ap33-1* and *ap65-1*, which are representative of other adhesin gene families [11]. Numbers on the left of panel (a) refer to size standards derived from the 100 bp ladder DNA (GIBCO-BRL). (II) Southern analysis of restricted genomic DNA of the original *T. vaginalis* isolate T016 from which the cDNA library was made as well as of other numerous representative isolates used previously [8, 11–13]. Genomic DNA was digested with *Eco*RI, which cuts outside each of the *ap51* genes, as confirmed by sequence analysis (Fig. 2) and restriction mapping (data not shown). Blots were probed with ^{32}P-labeled, purified AP51.2 cDNA or with individual gene specific oligo probes as shown in Part I. All procedures are as described in Materials and methods. As expected, because of the cross-hybridization among the adhesin genes, more complex banding patterns were seen when the AP51.2 cDNA was used as a probe. Importantly and not unexpectedly, the three gene-specific probes gave a collective pattern identical to that of AP51.2 cDNA. Each *ap51* gene appeared to exist as a single copy among all isolates tested, and the *ap51-1* gene exhibited a minor restriction fragment-length polymorphism. Numbers on the left refer to DNA size markers that were a combination of the 100 bp ladder and *Hin*dIII-digested λ DNA (GIBCO-BRL).

Distinguishing features of the three distinct AP51 proteins

Figure 6 depicts the sequence alignment of all three proteins. The predicted protein sequence of AP51-1 has 93% and 94% identity with AP51-2 and AP51-3 adhesins, respectively. Despite difficulties in obtaining complete 5′ ends of *ap51-2* and *ap51-3*, nucleotide sequence obtained through 5′ amplification confirmed the differences in amino acids at positions 3 and 4 and, therefore, that each AP51 protein had similar yet distinct putative leader sequences. We were unsuccessful, however, in attaining the complete

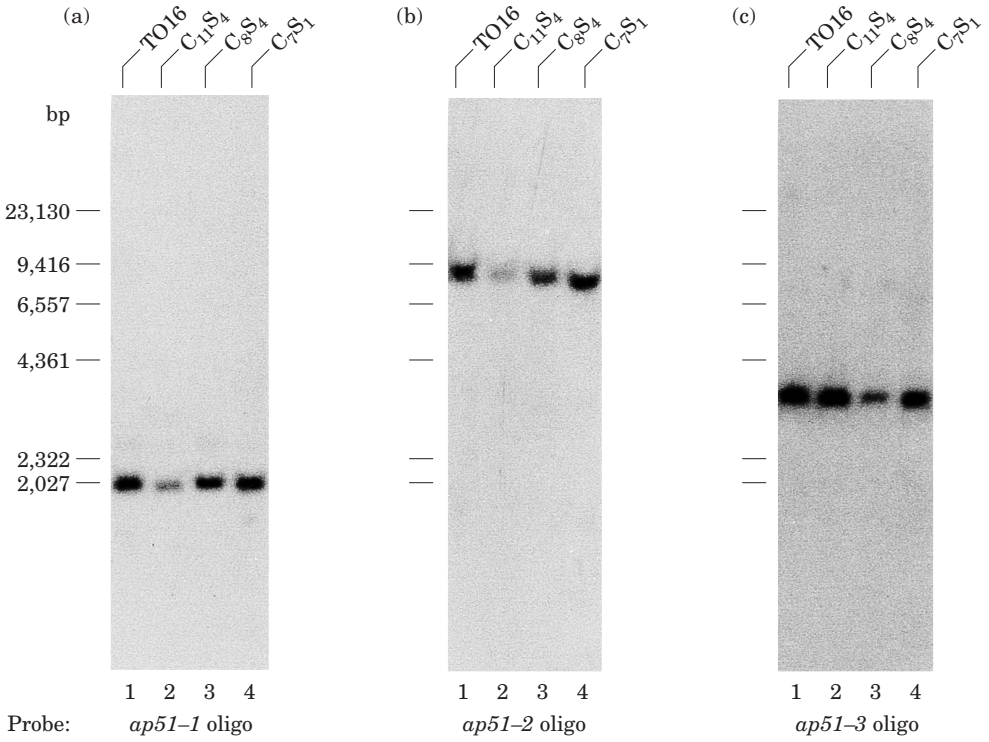

Figure 4. Southern analysis showing the existence of *ap51-1* (a), *ap51-2* (b), and *ap51-3* (c) genes among agar clones of individual trichomonads. Southerns were performed on genomic DNA of three representative agar subclones of *T. vaginalis* isolate T016 digested with *Eco*RI and probed with the gene-specific oligos used in Fig. 3. The patterns of hybridization on Zeta-Probe GT membranes were identical to those seen previously for the isolates (Fig. 3), indicating the non-allelic nature of these genes. Numbers on the left refer to the *Hin*dIII-digested λ DNA (GIBCO-BRL) used for size estimations.

5′ sequence and lacked 8 nucleotides and 4 nucleotides from the ORFs of *ap51-2* and *ap51-3*. However, N-terminal sequence from protein purified from the ligand assay [7, 8] (Materials and methods) gave multiple amino acids at only positions 3 and 4 after the initial methionine, both affirming the amino acid sequence at these positions and indicating that the first two amino acids (ML) are likely identical for all proteins.

Despite strong identity among the three AP51 proteins, AP51-3 had a pI of 8.11, further distinguishing it from AP51-1 and AP51-2, which had calculated pIs of 6.56 and 6.75, respectively. Computer hydropathy, although giving similar profiles for the three proteins (data not shown), revealed for each protein the three most probable antigenic sites. Of the three peptide sequences identified by hydropathy analyses, two of them, VEVKRE (amino acids 119-124) and EIRAEK (259-264), were common to all proteins (Fig. 6). The third antigenic peptide region was ARSPEE (34-40) for AP51-1, KLIRES (376-381) for AP51-2, and KQTGKD (101-106) for AP51-3 despite the fact that some of the sequences were found within the other AP51 proteins.

Two putative transmembrane helices were identified (boxed regions). One helix at position 276 to 293 (VACLVNGAGLAMATMDVI) was common to all three. The other helix was common to both AP51-1 and AP51-2 at position 330 to 353 (AILVNIFGGIVRCDMVAAGVIAAF). A consensus membrane-spanning domain (shaded amino acid sequence region) was identified for AP51-1 and AP51-2, but not for AP51-3 at position 335 (IFGGIVRCDMVAAGVIA). For both the transmembrane helix and membrane-spanning domain, only one amino acid change from alanine to arginine at position 341 disallowed these motifs for the AP51-3 protein.

Identity comparisons with known proteins

As shown in Figure 7, sequence comparisons revealed the three AP51 proteins had a 46% identity with the porcine β-succinyl coenzyme

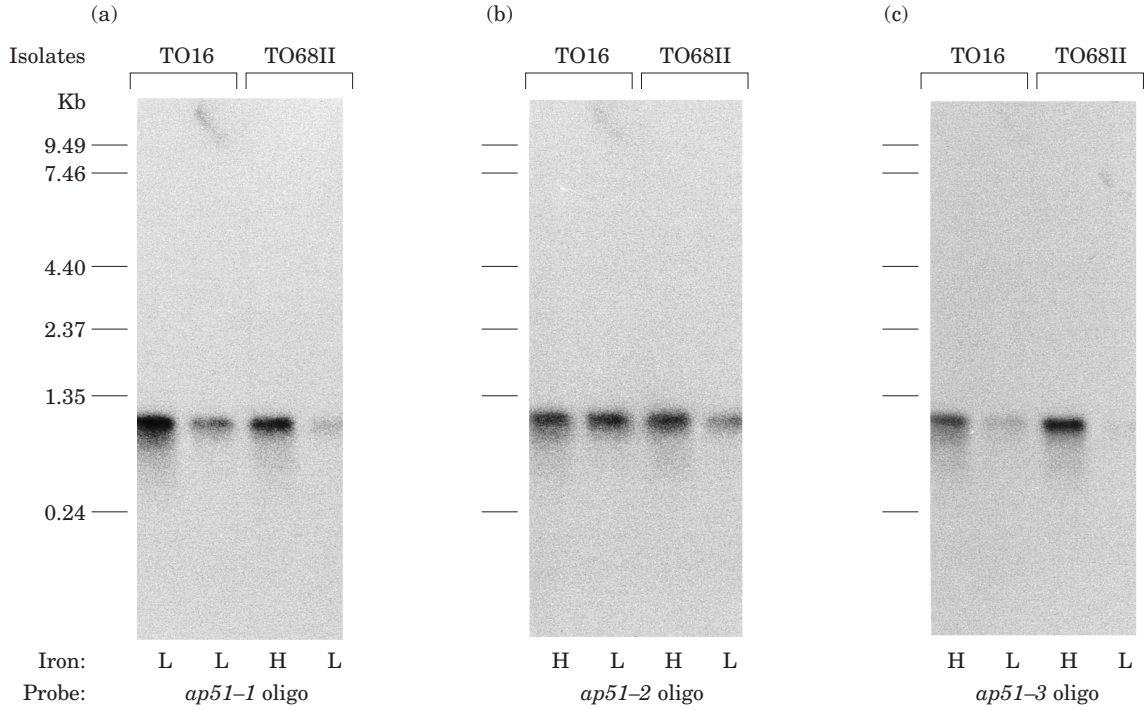

Figure 5. Northern analysis reveals that iron up-regulates expression of the *ap51-1* and *ap51-3*, but not *ap51-2*, genes in *T. vaginalis* isolates T016 and T068-II. Purified mRNA (3 μg) from two isolates used recently [12] grown in high- (lanes labeled H) and low-iron (lanes labeled L) medium [10] was electrophoresed in denaturing 1.2% formaldehyde agarose gels. After transferring the mRNA onto Zeta-Probe GT membranes, blots were probed with ^{32}P-labeled gene-specific probes that represented the antisense oligonucleotides to the original sequence used for Southerns (Fig. 3). Only longer exposure of X-ray film revealed *ap51-1* and *ap51-3* transcript in the low-iron-grown trichomonads for both isolates. Agar subclones as used in Figure 4 gave similar results, reaffirming expression and regulation by iron of each of the *ap51* genes among all trichomonads. The RNA molecular size markers (kb) (GIBCO-BRL) were used for size estimations.

A synthetase subunit (β-SCS). A more direct protein comparison highlighted five regions of unknown function (boxed areas) with strong identities between AP51 and β-SCS. Although the *ap51-2* sequence is identical to that of *T. vaginalis* β-SCS recently reported [24], the enzymatic activity of the reported sequence awaits experimental verification. It is also noteworthy that the antigenic sites, transmembrane helices, and membrane-spanning domain (Fig. 6) were not found within the known β-SCS sequences, despite the similarities.

Because of the homology with porcine β-SCS, we performed a competition experiment in which HeLa cells were pretreated with excess amounts of pure porcine SCS followed by addition of an extract of radiolabeled *T. vaginalis* proteins [Fig. 1, part (IIb)]. Addition of up to 2 mg of pure SCS had no effect on the amount of AP 51 (lanes 1 through 4) or any other adhesin protein binding to host cells. The same intensity of labeled AP51 was evident in the presence (lanes 2 through 4) and absence (lane 1) of β-SCS. In contrast and as shown earlier, unlabeled natural or recombinant AP51 readily decreased binding of the ^{35}S-labeled trichomonad protein [part (IIa)]. These data illustrate that, despite the homology of AP51 with β-SCS, sufficient protein differences exist that give AP51 receptor-binding function.

Discussion

This report presents evidence showing the existence of three non-allelic *ap51* genes, two of which are transcribed optimally under iron-replete medium conditions. Characteristics of the AP51 adhesin reinforce the emerging theme [19, 20]. That the adhesins are bifunctional, having both enzymatic and adhesive properties is conceivable based on precedence. This would seem to have significant consequences in con-

Figure 6. Alignment of the predicted amino acid sequences of the three AP51 proteins. The arrow denotes the putative cleavage site of the leader peptides (to the left of the arrow). The beginning of the AP51.2 clone originally isolated is indicated by the arrow to the right. The two boxed areas denote the computer-predicted transmembrane helices, and the shaded area within the box at position 335 is a possible membrane-spanning domain overlapping the transmembrane helix motif. Predicted antigenic sites for the three AP51 proteins are underlined. Xs at the translational start site for AP51-2 and AP51-3 are a reflection of the inability to obtain the complete nucleotide sequence (Materials and methods). However, the amino acids are most likely identical to those for AP51-1 for positions 1 and 2, and is either S or N for position 3, based on N-terminal sequence information obtained from purified proteins (Results).

siderations of parasite adaptations to the host environment. Because the AP65 [12, 13] and AP51 adhesin candidates are both members of multigene families, it is equally probable that recruitment of a new structural role may have resulted from gene duplication and subsequent separation of function [19, 20]. Whether all members of the multigene families have enzymatic and/or adhesive function awaits future experimentation.

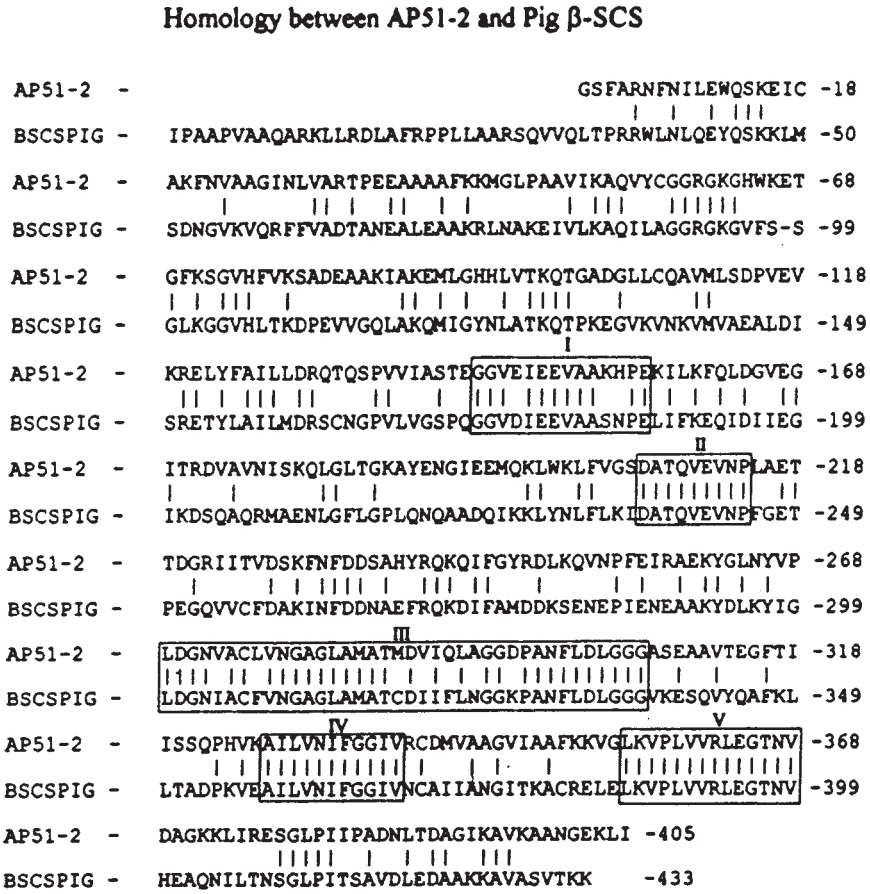

Figure 7. Extent of identity between AP51-2 and pig β-SCS. The four boxed areas represent regions with extensive identity, which is 46.78% between the two over the entire protein sequence. The identity between this protein and the β-SCS of *E. coli* [37] and *T. flavus* was 44.33 and 39.15%, respectively. No functionality has yet been assigned to a particular amino acid sequence, including the boxed areas, of β-SCS.

It is significant that expression of one of the *ap51* genes was not regulated by iron. This finding may also be relevant to the other adhesins, such as AP65 [11–13]. It is probable, since only three of the six *ap65* genes were examined by us, that some or all of the remaining *ap65* genes [25] may not be regulated by iron. The results shown here reinforce the idea that two isoforms of the same gene exist, one of which may be constitutively expressed while the second directly under the influence of iron. These results may further distinguish the genes that represent enzymes (constitutively expressed) from those that encode adhesins (environmentally regulated). Furthermore, features possibly acquired after gene duplication of some members of the family may have allowed for evolution of separate regulatory control mechanisms and for optimization of distinct protein functions. The presence of distinguishing features, among which include leader sequences, epitope differences, pIs, variations within membrane-spanning helices and transmembrane domains, 3′ site-directing sequences (zip codes) [27, 28], and, finally, 3′-end UTR destabilizing elements [21–23], may all dictate the ultimate location and function of adhesins within and on *T. vaginalis* organisms. Nonetheless, these results provide compelling evidence that it is essential to examine the nature of environmental signals that regulate important molecules, such as those involved in host/tissue parasitism.

There is precedence for post-translation regulation of these molecules. The gene expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), an enzyme reported to have a number of functions, including adherence [18, 19], has been shown to be regulated at the post-transcriptional level in rat tissue by adjusting mRNA abundance [28]. Although the mechanism of

regulation was not known, different levels of GAPDH gene expression correlated with differential polyadenylation. In group A Streptococci, the intracellular form of GAPDH functions as a metabolic enzyme, whereas the surface-located, ADP-ribosylated form functions as a ligand for plasmin [16]. Since AP65 and AP51, like GAPDH, may be related to metabolic enzymes recruited to the cell surface as adhesins, the finding that they may be similarly regulated by mRNA turnover, such as through destabilizing ATTTA motifs [21, 22], is significant for regulation of expression of virulence factors.

If any of the AP51 proteins is in fact the *T. vaginalis* β-SCS enzyme subunit [24], then, like the *ap65* genes that resemble malic enzyme [12, 13, 25], it is clear that the hydrogenosomal enzymes may be up-regulated by iron. To our knowledge this would be the first report for this finding for *T. vaginalis* metabolic enzymes. In support of a role for environmental factors signaling *T. vaginalis*, contact with VECs or HeLa cells was found to induce trichomonads to synthesize greater amounts of all four adhesins [9]. Importantly, we now have another multigene family distinguishable by gene-specific probes so that each gene can be evaluated for up-regulation of expression upon host colonization. The equal possibility exists that adhesin synthesis is regulated by the rate of transcript turnover, which would provide an important role for the destabilizing element ATTTA (Fig. 2), as reported recently [13], within the 3′-end UTR of some other adhesin genes. It is not insignificant, we feel, that this destabilizing element is not universally found among each member of the multigene family of adhesins. Knowledge of the precise role that this element has within the context of specific gene product expression in trichomonads should go a long way toward helping us understand the biology of this pathogen and the host-parasite interaction.

The recombinant AP51 protein contained epitopes immunocrossreactive with polyclonal antiserum against the natural surface *T. vaginalis* AP51 that bound to host cells [7, 8]. The original, truncated AP51.2 encoded a recombinant protein with function, as evidenced by its ability to bind to host cells [11] and to inhibit the binding of the natural trichomonad AP51 protein. These data suggest, therefore, that the receptor-binding epitope resides either central or toward the carboxy-terminal region, similar to the findings for AP65 [12]. That we have three members in the *ap51* gene family, each of which is expressed in individual organisms (Fig. 4), points once again to the importance of these genes to trichomonal cytoadherence and the overall host-parasite interrelationship.

Commercially available β-SCS neither interfered with *T. vaginalis* cytoadherence nor competed with trichomonad AP51 binding to host cells, providing compelling evidence for a role of the unique features of these proteins in adhesive function. These differences in function between the β-SCS porcine subunit sequences and the AP51 adhesins may not be surprising given the limited amino acid sequence similarities in parts of the protein that likely play a role in host cell surface binding. It is noteworthy that the putative transmembrane helices and membrane-spanning domains do not reside within the porcine β-SCS, emphasizing the significant differences between these proteins. We nonetheless considered the likelihood that one of the AP51 proteins may be the trichomonad β-SCS subunit. A recent paper presenting sequence of a putative *T. vaginalis* hydrogenosome protein was reported to code for the β-SCS subunit [24], and in this report, one gene (gene B) had a sequence identical to *ap51-1*. An additional partial cDNA sequence, called cDNA-A, was non-identical with both *ap51-2* and *ap51-3*, suggesting the possibility of yet another member in this multigene family. It must be carefully considered that this report did not show any function for the protein encoded by the reported β-*SCS-1* DNA sequence. Furthermore, the hypothesis that isolating metabolic enzymes from hydrogenosome vacuoles [24, 25] might equally permit for fractionation of vacuoles containing the trichomonad adhesins is attractive and testable, possibly through selective fractionation procedures. It is plausible that efficient transport of the four adhesins to the trichomonad membrane follows packaging into vacuoles. This hypothesis is supported by the established fact that synthesis and surface expression of the four adhesins occurs in a coordinated fashion [8, 10].

Materials and methods

Microorganisms and culture conditions

Trichomonas vaginalis T016N, a fresh clinical isolate, expressed high amounts of adhesins under iron-replete growth conditions [8, 9, 11]. The growth medium of Trypticase-yeast extract-maltose (TYM) with 10% heat-inactivated horse

serum (HIHS) was used, as before [28]. Where needed, the medium was supplemented with 250 μM ferrous ammonium sulfate-hexahydrate (Sigma Chemical Co., St Louis, MO, U.S.A.), prepared as a 100-fold stock solution in 50 mM sulfosalicylic acid [10]. Parasites for all experiments were grown to the late-logarithmic-phase of growth. Recombinant *Escherichia coli* INVαF′ (Invitrogen Corp., San Diego, CA, U.S.A.) harboring the phagemid vector pcDNAII (Invitrogen) with the cDNA clone AP51.2, which was known to encode a portion of the AP51 adhesin [11], was cultured in Luria-Bertani (LB) broth or LB agar plates with 60 μg/ml ampicillin [11–13]. Agar clones and subclones derived from single trichomonads of *T. vaginalis* isolate T016N and T068-II were isolated as detailed recently [29].

Nucleic acid isolation of *T. vaginalis*

Purified mRNA was obtained by FastTrack 2.0 as specified by the manufacturer (Invitrogen). Total genomic DNA from trichomonads was isolated as detailed recently [11, 12, 31]. Briefly, 10^8 parasites were lysed, and DNA was extracted with phenol-chloroform. After ethanol precipitation and re-suspension, the DNA was treated with RNase A and proteinase K.

Generation and isolation of cDNA

The construction and screening of a *T. vaginalis* cDNA expression library was as performed initially [11]. Recombinant *E. coli* colonies immunoreactive with polyclonal anti-AP51 serum previously generated and characterized [7, 8] were isolated and purified [32]. Analysis of inserts 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) [12, 31, 32].

DNA sequencing and analysis and sequence accession numbers

DNA sequencing was performed by the dideoxy chain termination method using the Sequenase 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, OH, U.S.A.), as recommended, and as previously described [12, 13]. Computer analysis of the sequence was carried out using PC/GENE (Release 6.8) (IntelliGenetics Inc., Mountain View, CA, U.S.A.). The nucleotide sequence of *ap51-1*, *ap51-2* and *ap51-3* have been assigned GenBank accession numbers U87093, U87094, and U87095, respectively.

5′ and 3′ rapid amplification of AP51 cDNA ends (RACE)

The Marathon cDNA Amplification kit (Clontech Lab. Inc., Palo Alto, CA, U.S.A.) was used as recommended by the manufacturer to generate both 5′ and 3′ sequence for the AP51 genes. Starting with 5 μg of partially purified mRNA, first-strand synthesis was performed using a lock-docking oligo (dT) primer. After second-strand synthesis, the double-stranded cDNA was ligated to the Marathon cDNA Adaptor. Amplification was performed using the Marathon adaptor primer and an internal AP51 primer (antisense for 5′ and sense for 3′ amplification). Figure 2 shows the primers (underlined) that were used in conjunction with the adaptor primer to obtain the three *ap51* genes. The internal primers were 51CO5E (antisense) and AP51P1R (sense) for *ap51-1*, AP51P1 (antisense) for *ap51-2*, and AP51P3 (antisense) and 51T (sense) for *ap51-3*. The PCR conditions were 25 cycles and 94°C for 30 s followed by 68°C for 3 min for all reactions except for primer 51T, which was 68°C for 5 min. All amplified products were analysed immediately after synthesis on 1% agarose gels, after which clones were subcloned directly into pCRII (Invitrogen).

Southern and Northern analysis

Southern analysis [33], used to analyse cDNA or trichomonad genomic DNA, was performed as described previously [11–13]. Hybridizations were carried out with purified, nick-translated cDNA inserts or end-labeled oligonucleotides used as probes [13, 31]. Transcripts were detected by performing Northern analysis of electrophoresed mRNA as previously described [11–13,]. Blots were probed with the end-labeled oligonucleotides specific for each of the *ap51* genes and referred to as AP51-1 oligo (specific for *ap51-1*), AP51-2 oligo (*ap51-2*) and AP51-3 oligo (*ap51-3*). Hybridization reactions using the oligonucleotides as probes was with the ExpressHyb Solution (Clonetech), as indicated by

the manufacturer. Otherwise, hybridization reactions were performed after transferring of DNA or mRNA onto Zeta-Probe GT membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.) as recently detailed [11–13, 34].

SDS-PAGE and immunoblotting of recombinant proteins

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been reported [7, 8, 35]. *E. coli* growing in LB broth were pelleted, suspended in electrophoresis dissolving buffer [35], and electrophoresed using 10% separating and 4% stacking gels. Gels were stained with Coomassie brilliant blue following electrophoresis. Duplicate gels after SDS-PAGE were transferred to nitrocellulose for immunoblotting [8, 10, 36]. Blots were probed with polyclonal rabbit anti-AP51 antiserum originally generated to the HeLa cell-enriched AP51 [8, 11]. This antiserum was specific to AP51 and did not immuno-crossreact to any other adhesins [8, 11]. Normal, prebleed rabbit serum was used as a negative control throughout.

Preparation of French press *E. coli* extracts

Recombinant *E. coli* extracts were prepared by subjecting the bacteria to the French pressure cell for lysis as described before [11]. This bacterial lysate was clarified by centrifugation at 17 500 *g* for use in the ligand assay as described below.

The ligand assay

A ligand assay has been extensively described by us [7, 8, 10, 12] and was used to analyse the natural and recombinant proteins. Briefly, French-press bacterial lysate (1 ml) was incubated with 10^6 fixed HeLa cells for 18 h at 4°C before the cells were washed to remove non-specifically associated bacterial proteins. Bacterial proteins avidly bound to HeLa cell surfaces were then eluted by boiling in electrophoresis dissolving buffer [35], after which released proteins were electrophoresed for immunoblotting. To further show function for the recombinant protein, a competition experiment was performed [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 labeled with EXPRE^{35}S^{35}S-Protein labeling mix [11, 12] and then washed. We have previously found that AP51 is readily radiolabeled under these conditions [8, 10, 11]. Radiolabeled proteins associated with HeLa cells were eluted by boiling in electrophoresis dissolving buffer for 3 min [35], and analysed similarly by electrophoresis. Gels were stained and prepared for fluorography. Cell lysates of *E. coli* containing the plasmid without any cDNA inserts were used identically as controls.

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