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The proteins secreted by *Trichomonas vaginalis* and vaginal epithelial cell response to secreted and episomally expressed AP65

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Summary

We showed recently that contact of human vaginal epithelial cells (VECs) by *Trichomonas vaginalis* and incubation with trichomonad proteins in conditioned medium induced expression of VEC genes. We performed 2-D SDS-PAGE followed by MALDI-TOF to identify the major secreted proteins. Based on protein abundance and separation of spots in 2-D gels, 32 major secreted proteins were examined, which gave 19 proteins with accession numbers. These proteins included known secreted cysteine proteinases. In addition, other secreted proteins were enzymes of carbohydrate metabolism, adhesin protein AP65, heat shock proteins, thioredoxin reductase and coronins. We confirmed that the secreted trichomonad proteins induced expression of VEC genes, including interleukin 8 (IL-8), COX-2 and fibronectin. Purified AP65 added to VECs had a pronounced effect only on IL-8 gene expression, which was inhibited in the presence of 12G4 monoclonal antibody to AP65. Moreover, AP65 expressed episomally within epithelial cells was found to enhance the expression of IL-8 and COX-2. This may be the first report of analysis of the secreted proteins of *T. vaginalis* and of the host epithelial cell response to these proteins and to the prominent adhesin AP65.

Introduction

Trichomonas vaginalis causes trichomonosis, the number one, non-viral sexually transmitted infection (STI) worldwide. This STI is considered a health disparities disease (Sorvillo *et al*., 1998) that remains poorly studied. Infection by *T. vaginalis* is associated with serious adverse health consequences to women that include infertility (El-Shazly *et al*., 2001), atypical pelvic inflammatory disease (Moodley *et al*., 2002), preterm birth and low birth weight infants (Cotch *et al*., 1997), and predisposition to cervical neoplasia (Viikki *et al*., 2000). Trichomonosis among men can cause non-chlamydial, non-gonococcal urethritis (Bennett *et al*., 1989; Bakare *et al*., 1999) and, more recently, serum antibody in men to *T. vaginalis* was found to be related with prostate cancer (Sutcliffe *et al*., 2006). For both men and women, trichomonosis increases predisposition to HIV seroconversion (Guenthner *et al*., 2005; Mason *et al*., 2005; Rughooputh and Greenwell, 2005). These sequelae are especially significant given the astounding high rates and long-term duration of this STI in both women and men (Van Der Pol *et al*., 2005).

The complex profile of both the parasite and host responses following cytoadherence and during infection remains poorly characterized. We showed that brief contact of trichomonads with vaginal epithelial cells (VECs), but not HeLa cells, produced dramatic changes in parasite morphology, as evident from the parasite transformation from an ellipsoid to amoeboid form (Arroyo *et al*., 1993), suggesting host-specific signalling of parasites by VECs. Five different parasite surface proteins (AP120, AP65, AP51, AP33 and AP23) mediate adherence (Arroyo *et al*., 1992; Moreno-Brito *et al*., 2005), and genes encoding adhesins are upregulated in expression during attachment to VECs (Garcia *et al*., 2003; Kucknoor *et al*., 2005a). In a separate study using different forms of parasites grown in culture flasks, α-actinin was shown to be overexpressed in amoeboid parasites compared with batch-cultured ellipsoid trichomonads (Addis *et al*., 1998).

While overall immune responses during trichomonosis are largely unknown, high levels of interleukin-8 (IL-8) and leukotreine B4 (LTB4) have been found in the vaginal secretions from symptomatic patients with trichomonosis (Shaio *et al*., 1994; 1995; Shaio and Lin, 1995). There are also reports of IL-8 produced in response to *T. vaginalis* stimulation by human neutrophils (Ryu *et al*., 2004) and human monocytes (Shaio *et al*., 1995). Further, *in vitro* studies have revealed that IL-8 production is regulated through NF-κB and MAP kinase signalling pathways. Nonetheless, little is known about the host responses resulting immediately from infection by *T. vaginalis*.

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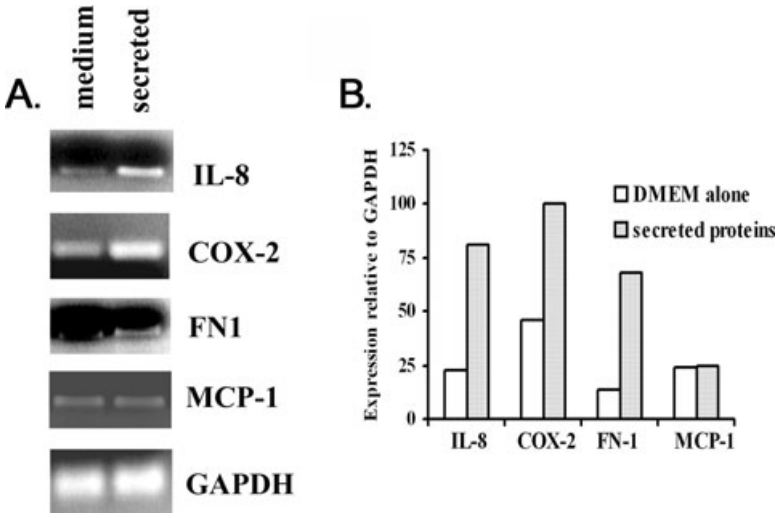

Fig. 1. Representative results from a semiquantitative RT-PCR experiment showing induction of host gene expression patterns stimulated by *T. vaginalis* secreted proteins. Total RNA from MS-74 VECs incubated with growth medium alone or with secreted proteins was isolated (*Experimental procedures*). RNA was reverse-transcribed using oligo(dT) primer and PCR performed using gene-specific primers.
A. Showing the RT-PCR products separated on ethidium bromide (EtBr)-stained gels after electrophoresis in 2% agarose.
B. Illustrating the extent of gene expression pattern relative to the housekeeping GAPDH gene used as a control. The values were obtained by scanning the intensity of bands from pictures of the gels in A using the Scion image beta program. The expression for each gene was relative to baseline density for GAPDH plotted on the graph. This experiment was repeated on four separate occasions with similar results.

Justifiably, studies have focused on examining the secreted proteins of microbial pathogens (Bumann *et al*., 2002; Knudsen *et al*., 2005; Medina *et al*., 2005; Trost *et al*., 2005). The secreted proteins may mediate important pathogen–host interactions, including eliciting symptoms, immune evasion and pathogenesis. Much remains to be learned about the trichomonad virulence factors involved in symptomatology and pathogenesis among some women and men. In a recent study, we showed that *T. vaginalis* adherence to VECs upregulated expression of numerous genes that may play a role in pathogenesis (Kucknoor *et al*., 2005b). Moreover, trichomonad proteins present in conditioned medium also induced expression of VEC genes. Therefore, because secreted proteins may promote survival of *T. vaginalis* in the vagina and to better understand the secreted proteins of *T. vaginalis* that interact directly with the vaginal epithelium during infection, we wanted to characterize these proteins. By two-dimensional (2-D) electrophoresis and MALDI-TOF mass spectroscopy we identified secreted proteins to be proteases, the prominent adhesin AP65, heat shock proteins (HSPs), enzymes of carbohydrate metabolism, thioredoxin reductase and coronins. We further show that secreted proteins induce expression of IL-8, COX-2 and FN-1 in VECs. Purified extracellular AP65 induced expression of only IL-8, and, for the first time, we demonstrate the episomal expression in HeLa cells of AP65 and increased synthesis by host cells of IL-8 and COX-2, suggesting a major role of this adhesin in host responses.

Results

Upregulation of host genes induced by secreted medium

We wanted to confirm that secreted *T. vaginalis* proteins induced expression of VEC genes, as before (Kucknoor *et al*., 2005b). We therefore selected the genes encoding the cytokine IL-8, cyclooxygenase-2 (COX-2), fibronectin (FN1) and monocyte chemoattractant protein (MCP-1). The levels of specific mRNAs of known genes were analysed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). IL-8, COX-2 and FN-1 are known to be elevated by conditioned growth medium (Kucknoor *et al*., 2005b). We normalized expression of each gene to VEC glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared expression of immortalized MS-74 VECs incubated with the secreted proteins to control VECs handled identically except without added trichomonal extract or with control medium. RT-PCR products after agarose gel electrophoresis and staining are shown in Fig. 1A. Except for the MCP-1 gene, the genes for IL-8, COX-2 and FN1 had two- to threefold increased amounts of RT-PCR product compared with controls. As before, the MCP-1 gene was not induced by the secreted proteins, and this suggests that only parasite contact with VECs (Kucknoor *et al*., 2005b) elicits a complete host response. As a control, medium alone gave a basal level expression of the four genes. These data suggest that secreted proteins and/or contact by *T. vaginalis* (Kucknoor *et al*., 2005b) induce host cell responses. Figure 1B

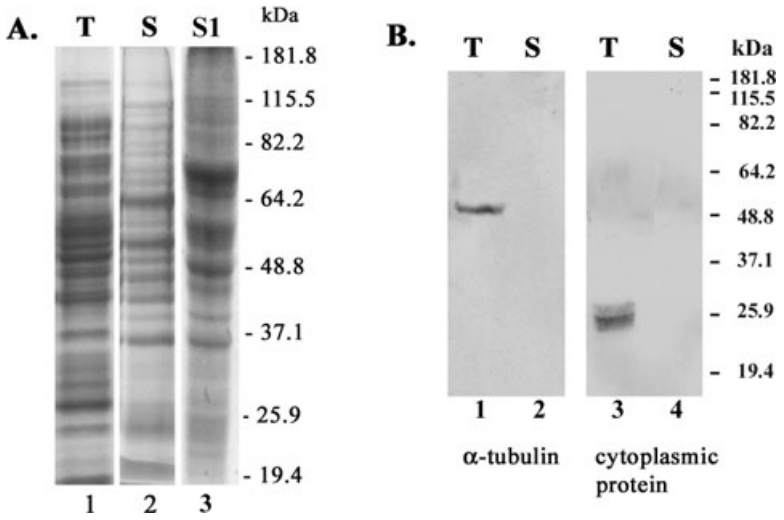

Fig. 2. The secreted proteins of *T. vaginalis*. Preparations of total protein from whole cell lysates (T), secreted proteins (S) and secreted proteins by parasites adherent to VECs (S1) were separated by SDS-PAGE using 10% acrylamide gels.
A. Presenting the complex patterns of proteins after Coomassie brilliant blue staining of gels.
B. Showing the immunoblots probed with mAbs to α-tubulin and L64 to a 30 kDa cytoplasmic protein. Numbers on the right side refer to electrophoretic mobilities of protein standards in kDa.

presents the levels of increased expression relative to GAPDH as quantified by the Scion image beta program, further affirming the net increased expression compared with the controls. These data show that trichomonad proteins from cell-free supernatants signal for elevated expression of VEC genes.

Visualization and evaluation of secreted proteins

To visualize and evaluate the quality of the secreted proteins, we then electrophoresed the proteins by SDS-PAGE. Figure 2A shows protein profiles after electrophoresis of total trichomonal lysate (T, lane 1), and of TCA-precipitated secreted proteins (S), and of TCA-precipitated proteins secreted by parasites adherent to VECs (S1). Not unexpectedly because of the known upregulated expression of trichomonad proteins by contact with VECs (Kucknoor *et al*., 2005a), the intensity of some secreted proteins was increased after contact compared with the secreted proteins of parasites alone. Under these conditions, control supernatant from VECs alone had few detectable proteins (data not shown). As a control to monitor contamination of secreted proteins resulting from trichomonal lysis, duplicate gels were blotted onto nitrocellulose (Fig. 2B) and probed with mAbs specific to α-tubulin and L-64 to a 30 kDa cytoplasmic protein (Alderete *et al*., 1987). Proteins corresponding to α-tubulin and to the 30 kDa cytoplasmic protein were detected only in the trichomonal lysate (lanes 1 and 3) but not in the secreted protein preparation (lanes 2 and 4), indicating maintenance of parasite integrity during growth.

Two-dimensional SDS-PAGE of secreted proteins and protein identification

Figure 3 presents a representative 2-D profile of total secreted proteins. This pattern after 2-D electrophoresis was considerably less complex than that seen in total protein preparations (Alderete and Garza, 1984; Alderete *et al*., 1986; Khoshnan *et al*., 1994). Protein spots arbitrarily chosen based on the protein abundance and clear spot separation for MALDI-TOF mass spectroscopy analysis are indicated by the numbered arrows. Table 1 lists the proteins, and as can be seen, a total of 32 spots analysed yielded the identity of 19 proteins. We had difficulty identifying proteins < 30 kDa because of the presence of multiple species within the digested peptides used for Mascot analysis. Among the major secreted proteins were several enzymes of carbohydrate metabolism, two cysteine proteinases, the adhesin AP65, an endoplasmic reticulum HSP, cytoplasmic HSP, an enzyme involved in arginine metabolism, thioredoxin reductase and cytoskeleton-related proteins actin and coronins. In the spot 4 region of the 2-D gel, there are other AP65 proteins that have been recently characterized on 2-D gels that react with the 12G4 mAb (Garcia *et al*., 2003). These data are consistent with our earlier report on the complex profile of secreted or released proteins in cell-free culture supernatants under conditions where no lysis is detected (Alderete and Garza, 1984).

Upregulation of host genes by purified AP65

As reported by others (Addis *et al*., 1997), AP65 was detected in the secreted protein preparation (Table 1). Because adherence is mediated by the prominent adhesin AP65 (Garcia *et al*., 2003; Mundodi *et al*., 2004), and parasite contact induces signalling of VEC genes (Kucknoor *et al*., 2005b), we hypothesized that purified native AP65 signalled VECs for induced expression of genes. Figure 4A shows the immunoblots probed with 12G4 mAb to AP65 purified from a trichomonad lysate (lane 1) versus the secreted proteins (lane 2). VECs were incubated with purified AP65 for 2 h, and RNA from the

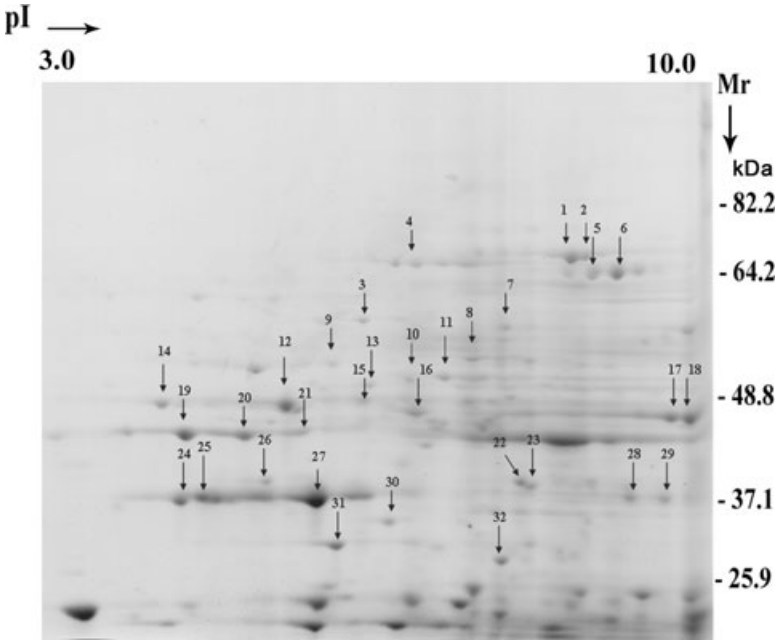

Fig. 3. The secreted proteins of *T. vaginalis* analysed by 2-D SDS-PAGE. The proteins were precipitated by TCA (*Experimental procedures*) for electrophoresis, and visualization of spots was by Coomassie brilliant blue staining of gels. Thirty-two proteins in the range of pH 3–10 were randomly chosen for characterization and identification by MALDI-TOF mass spectroscopy and are indicated on the gel by numbered arrows.

VECs was isolated. Figure 4B presents agarose gels and Fig. 4C shows the Scion image quantification of semi-quantitative RT-PCR products after electrophoresis. Five-fold higher amounts of IL-8 transcript were detected in VECs incubated with AP65 compared with control. Incubation of VECs with purified AP65 in the presence of 12G4 mAb resulted in control amounts of IL-8 transcript (Fig. 4B, lane 3), suggesting IL-8 induction by AP65 signalling. Interestingly, there was only a slight increase in band intensities after agarose electrophoresis (Fig. 4B) and Scion image quantification (Fig. 4C) for genes encoding for COX-2 or FN1, which were not reduced in the presence of 12G4 mAb (not shown), perhaps suggesting non-specific effects. The GAPDH gene control confirms that equal amounts of RNA were added to the samples for RT-PCR (Fig. 4B). We then confirmed that elevated levels

Table 1. List of proteins corresponding to numbered spots in two-dimensional SDS-PAGE analysis of secreted proteins.[a]

Spot[a]	Protein ID[b]	MW	pI	Accession number	Peptides[c]
1, 2, 3[d]	Endoplasmic reticulum HSP 70	68253	5.1	3510734	24, 17, 26
4	Adhesion protein AP65-1	63269	7.2	687630	14
5, 6	Cytoplasmic HSP 70	70259	5.5	3510736	21, 23
7	Unknown	54346	5.5	851069	12
8	Enolase	51283	5.8	58429954	11
9, 10, 11	Coronin	37427	5.4	7160326	12, 21, 8
12	Coronin	48174	5.5	7160324	11
13	Chaperonin subunit CCT zeta	57974	6.0	10567604	13
14	Malic enzyme	42501	5.9	33243008	11
15	Glyceraldehyde-3-phosphate dehydrogenase	39085	6.8	539406	15
16	Elongation factor 2				21
17, 18	Actin	40680	5.2	1480822	19, 11
19–21	Alcohol dehydrogenase 1	34171	6.3	21217455	17, 12, 10
22, 23	Cysteine protease	34641	6.4	452294	21, 16
24–27	Fructose-1–6-biphosphate aldolase	36239	5.8	14719270	33, 21, 4
28	Cysteine protease	31164	6.6	452296	11
29	Unknown	38437	5.5	7677551	8
30	Carbamate kinase	33906	5.6	4105707	17
31, 32	Thioredoxin reductase	32348	6.0	23095907	17

a. The numbered protein designations are those presented in Fig. 2.
b. Protein ID refers to the known protein identity determined by Mascot search using Swiss-Prot database.
c. Peptides refers to number of peptide digests used in the analyses and multiple numbers indicates the peptides used for identifying the proteins that gave multiple spots in the two-dimensional analysis.
d. Multiple numbers indicate spots where the MALDI-TOF analysis gave amino acid sequences to identical proteins with the same accession numbers.
ID, identity of protein; pI, isoelectric point; HSP, heat shock protein.

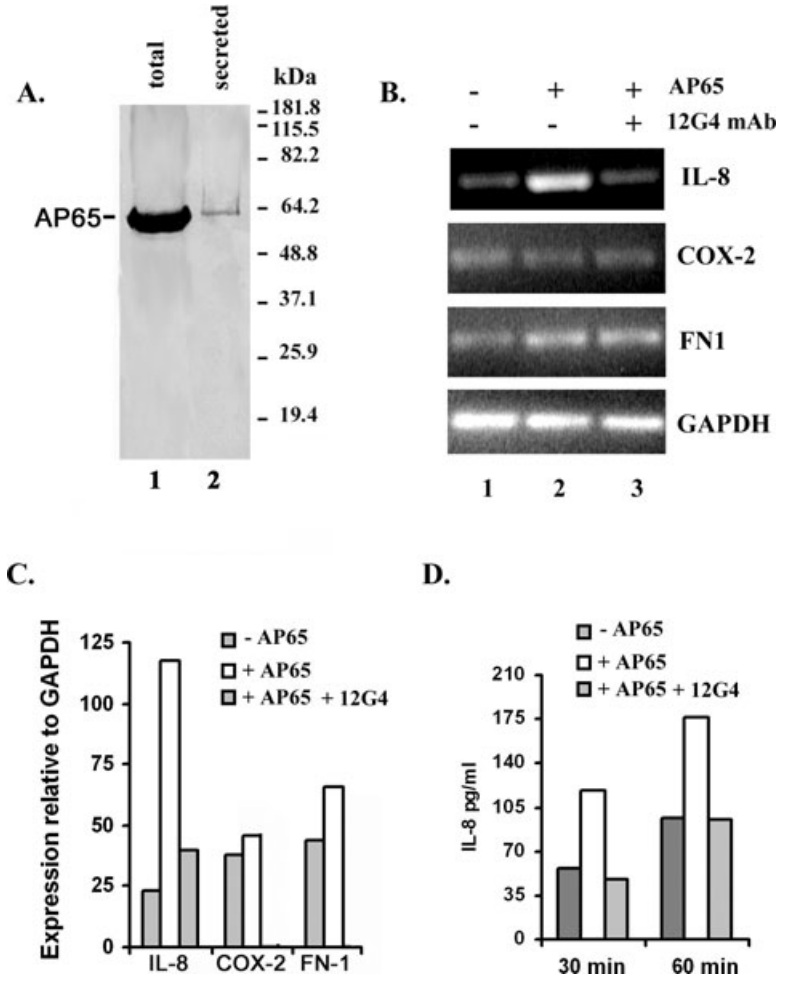

Fig. 4. Induction of host gene expression by addition of purified AP65 to VECs.
A. Showing the detection of AP65 in total protein lysates of *T. vaginalis* (lane 1) versus the secreted proteins (lane 2). Lysates were prepared from whole cells and secreted supernatant as described in *Experimental procedures* prior to SDS-PAGE and blotting onto nitrocellulose membranes. The blot was probed with the 12G4 mAb to AP65 (Garcia *et al*., 2003). The numbers indicate the molecular weight standards in kDa.
B. Showing representative results from one of four experiments. EtBr-stained bands after agarose electrophoresis of RT-PCR products for the four genes show the increased expression of IL-8 transcript (lane 2). GAPDH is included as the internal control unaffected by AP65 and to show that identical amounts of RNA were added to each reaction.
C. Illustrating quantitatively the gene expression pattern relative to the GAPDH gene as analysed by the Scion image beta program.
D. Showing the effect of AP65 on IL-8 production. Samples included confluent monolayers of control VECs or VECs stimulated with purified AP65. Additionally, VECs were treated with AP65 and 12G4 mAb. At selected times after incubation, supernatants were collected and assayed for IL-8. Four independent experiments were carried out and shown here is a representative experiment with the mean of quadruplicate samples. Samples did not vary more than 5% of the mean.

of IL-8 mRNA correlated with amounts of IL-8 (Fig. 4D). We quantified IL-8 by enzyme-linked immunosorbant assay (ELISA) (*Experimental procedures*) and found 176 pg ml^{-1} after 60 min incubation with AP65, which was reduced to control amounts (94 pg ml^{-1}) in the presence of 12G4 mAb. A similar trend was seen for the 30 min time point. These data suggest strongly that AP65 signals VECs for increased IL-8 gene expression.

Transient transfection of T. vaginalis *AP65 in HeLa cells*

We performed indirect immunofluorescence using 12G4 mAb to determine if AP65 is internalized after incubation with purified AP65 or adherence by *T. vaginalis*. Figure 5A shows that AP65 is in fact detected in permeabilized VECs in both conditions (panels a1 and a2). Similar fluorescence is neither seen with non-permeabilized VECs after removal of adherent organisms nor with VECs alone (panel a3). Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody with or without treatment with 12G4 mAb had no fluorescence in controls (panel a3). Based on this result, we hypothesized that transfection with AP65 would provide additional evidence and an alternative way to assess its role in host cell signalling for increased expression of genes without interference by other factors, as has been done in another microbial model (Kim *et al*., 2005). Therefore, we transfected HeLa cells and confirmed episomal synthesis of AP65 by immunoblotting and probing with 12G4 mAb. AP65 is detected on nitrocellulose blots of total proteins of transfected HeLa cells after SDS-PAGE (Fig. 5B, lane 2) compared with no bands in control cells or cells with plasmid without insert (lane 1). The multiple bands were the result of partial degradation of AP65 by HeLa cells. Figure 5C illustrates the intensity of RT-PCR products after agarose electrophoresis and staining to detect the transcript levels of the three select genes, as above (Fig. 4). Greater than twofold expression was apparent for both IL-8 and COX-2, but not FN-1, as evident by both intensity of bands and Scion image scan comparisons (Fig. 5C and D). These data suggest different signalling pathways depend on the presentation of parasite proteins.

As we detected higher transcript levels of IL-8 and COX-2 mRNAs in AP65-transfected HeLa cells, it was

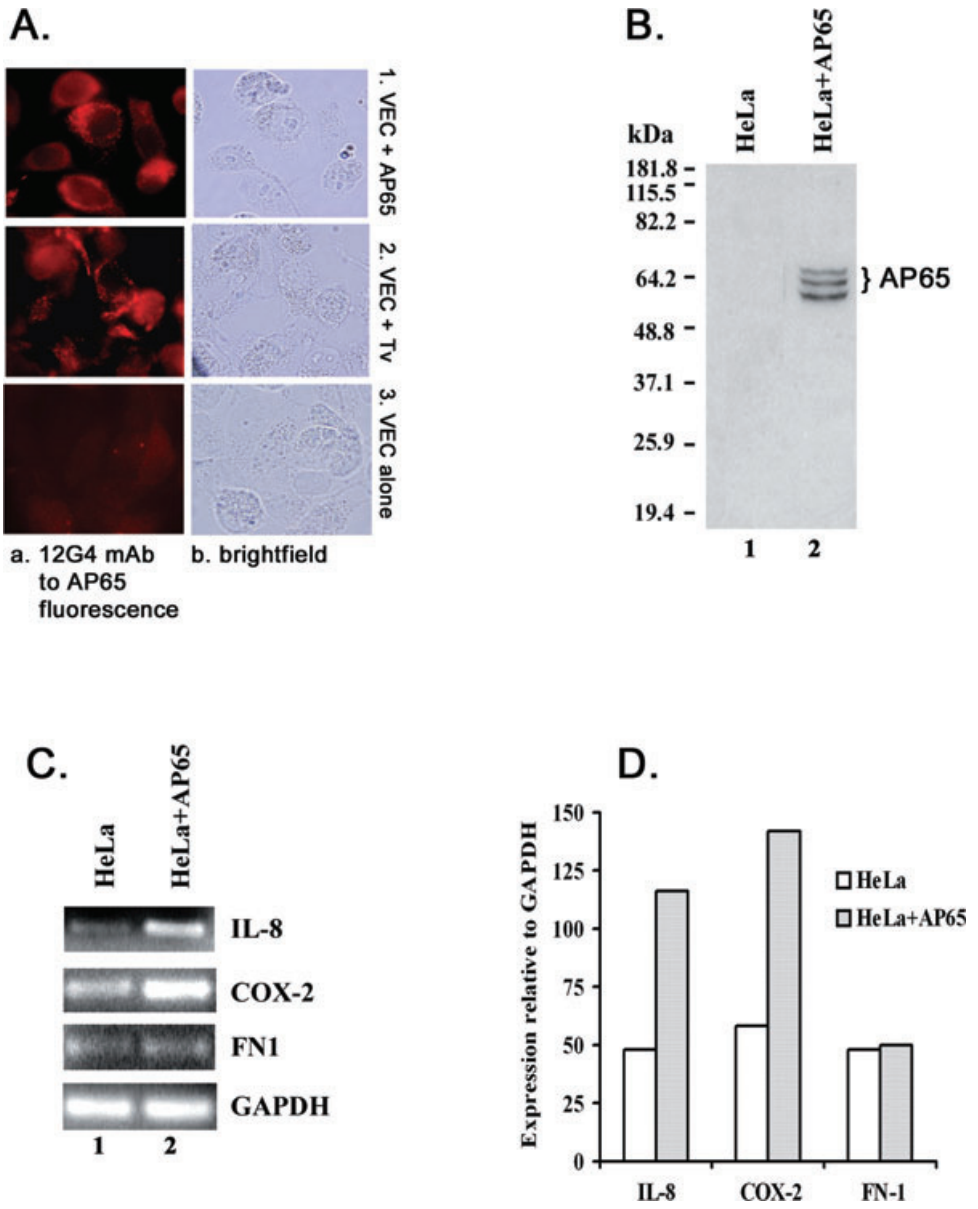

Fig. 5. Detection of AP65 in VECs incubated with purified AP65 and live trichomonads (A and B) and induction of host gene expression in HeLa cells expressing AP65 (C and D).
A. Shows the photomicrographs of VECs from immunofluorescence using 12G4 mAb. VECs were grown on chambered culture slides and incubated with purified AP65 (panel a1) or with live *T. vaginalis* organisms (panel a2) for 40 min and washed well (*Experimental procedures*). VECs were fixed and permeabilized before incubation with mAb. The immunostained cells were observed by microscopy in oil immersion with a final magnification of 1000×.
B. Showing the nitrocellulose blot after SDS-PAGE of total proteins from control (lane 1) and transfected HeLa cells (lane 2) probed with 12G4 mAb. HeLa cells were transiently transfected with the gene encoding *ap65-1*. The multiple bands are the result of degradation of AP65 in the cells. No immunocrossreactive proteins were ever detected in immunoblots of control HeLa cell total proteins or control cells and cells transfected with plasmid without insert.
C. Illustrating the PCR products after agarose gel electrophoresis and EtBr staining of the transcripts of the four genes of HeLa cells episomally expressing AP65.
Part D presents quantitative Scion image scans relative to GAPDH.

important to confirm elevated amounts of protein. We performed immunofluorescence to detect AP65 and COX-2 in the transfected HeLa cells. As seen in Fig. 6A, the synthesis and cytoplasmic localization of AP65 within transfected cells was evident (panel 1b), and COX-2 was readily visualized in the perinuclear region in transfected HeLa cells (panel 2b). Neither control HeLa cells labelled individually with mAbs to AP65 or COX-2 nor transfected

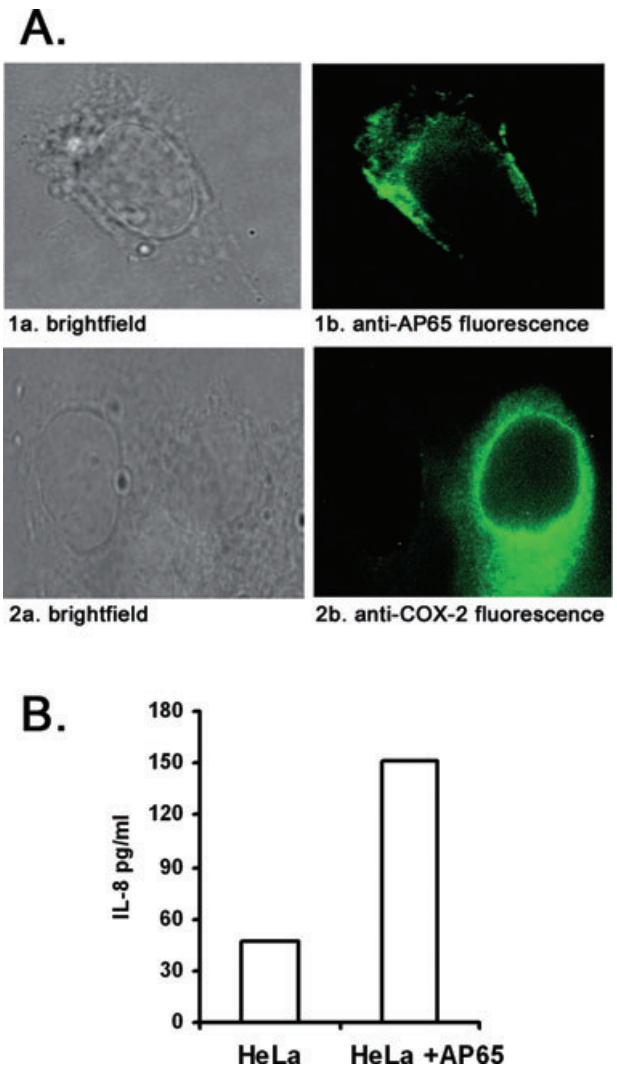

Fig. 6. Detection of AP65, COX-2 and IL-8 protein expression in AP65 transiently transfected HeLa cells.
A. Shown is of HeLa cells transfected with the plasmid, and after 24 h, transfected cells were washed well and incubated with either 12G4 mAb to AP65 (panel 1b) or COX-2 mAb (panel 2b) followed by FITC-conjugated anti-mouse secondary IgG antibody. The immunostained cells were observed by oil immersion microscopy at 1000 × final magnification. Panels 1a and 2a are brightfield microscopy of the identical cells presented in the fluorescence panels. No fluorescence was detected in the presence of either mAb or with secondary antibody in control HeLa cells.
B. Showing the quantification of IL-8 by ELISA (*Experimental procedures*) in culture supernatants of transiently transfected HeLa cells. Four independent experiments were carried out and shown here is a representative experiment with the mean of quadruplicate samples. Samples did not vary more than 5% of the mean.

HeLa cells without primary antibody handled identically had detectable fluorescence. Likewise, cells transfected with plasmid without insert were non-reactive by fluorescence. Finally, we then showed a threefold increase in the amount of IL-8 synthesized in transfected cells compared with the control (Fig. 6B). These data demonstrate that upregulation of IL-8 and COX-2 gene expression is related to synthesis of AP65 in the transfected cell.

Discussion

The non-self-limiting nature of trichomonosis occurs in the exceedingly complex and constantly changing urogenital tract of women. The menstrual cycle with the fluctuations in pH, iron and other nutrients and the desquamation of the VECs may represent external cues by which the infecting *T. vaginalis* organisms respond. Trichomonads must penetrate the mucus layer (Lehker and Sweeney, 1999) before contact with VECs (Arroyo *et al*., 1992) and the possible penetration into the basement membrane for binding to extracellular matrix proteins (Alderete *et al*., 2002). Trichomonal cytotoxicity of vaginal and cervical epithelial cells and the vaginal discharge following infection may be critical obstacles for successful host colonization by the parasite. It is known that secretions of patients contain numerous trichomonad proteins, including high molecular weight immunogenic proteins (Alderete *et al*., 1991) and cysteine proteinases (Alderete and Provenzano, 1997). Further, that numerous proteins are readily released and/or secreted during growth and multiplication without lysis of organisms has been established (Alderete and Garza, 1984). Recently, we showed that parasite contact with VECs induced expression of numerous VEC genes (Kucknoor *et al*., 2005b). Further, we found that spent supernatant from overnight-grown batch culture induced expression of genes after incubation with immortalized VECs as did live trichomonads. Therefore, it seems reasonable to hypothesize that secreted proteins are important mediators of host and parasite responses to infection that may promote either or both parasitism and pathogenesis.

Given the complex total protein composition of *T. vaginalis* by 2-D analysis (Alderete *et al*., 1986; Provenzano and Alderete, 1995) and the characterization of proteins released by trichomonads into cell-free culture supernatants (Alderete and Garza, 1984), we predicted that the profile of secreted proteins would be complex. From the complex 2-D patterns (Fig. 3), 32 major spots were characterized, which represented 19 unique proteins with corresponding accession numbers (Table 1). Most of these spots represent proteins with molecular weights to known sequences, indicating that, despite the known presence of cysteine proteinases, there was diminished or no degradation of the secreted proteins. Importantly, the proteins were not released due to damaged or lysed organisms, as before (Alderete and Garza, 1984), indicating that the 2-D patterns are an accurate portrait of the overall secreted proteins.

It is intriguing that six secreted proteins among those characterized are metabolic enzymes, and this includes AP65 with identity to the decarboxylating malic enzyme (Engbring *et al*., 1996). This finding is in agreement with the work of others on the release of AP65 in culture supernatants (Addis *et al*., 1997). Indeed, that this adhesin-enzyme, and not other proteins of the hydrogenosome organelle, is a member of the secreted protein family supports the fact that lysis is not responsible for proteins in growth medium. This confirms that AP65 is not solely a protein of the hydrogenosome organelle, and in fact it has been established that in fresh clinical isolates, iron upregulates expression of AP65 (Lehker *et al*., 1991) and other adhesins, and also modulates compartmentalization of these proteins outside the hydrogenosome organelle (Garcia *et al*., 2003).

More recently, we showed the trichomonad enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), among others, to be upregulated in expression following cytoadherence (Kucknoor *et al*., 2005a). Interestingly, GAPDH, fructose bis-phosphate aldolase (FBA), enolase and malate dehydrogenase have also been identified in the secreted proteomes of *Plasmodium falciparum* (Vincensini *et al*., 2005) and *Schistosoma mansoni* (Knudsen *et al*., 2005). Moreover, FBA, and GAPDH are highly immunogenic proteins effective in eliciting protection against *Streptococcus pneumoniae* (Ling *et al*., 2004), *S. mansoni* (Argiro *et al*., 2000) and *Onchocerca volvulus* (McCarthy *et al*., 2002). The literature is now replete with reports of microbial pathogens with anchorless, surface-associated metabolic enzymes (SAEs) (Alderete *et al*., 2001), and SAEs have functional diversity.

Microbial pathogens secrete proteins involved in the modification of disulphide bonds. *Helicobacter pylori*, for example, appears to modulate the disulphide bonds in its surroundings (Bumann *et al*., 2002), and, as suggested, the secretion of thioredoxin may play a role through changing the viscosity of mucus (McGee *et al*., 2006). We now show that thioredoxin reductase that may likewise be involved in the modification of protein disulphide bonds is secreted. Protein disulphide isomerase (PDI) was also found to be upregulated in trichomonads after contact with VECs (Kucknoor *et al*., 2005a). Thus, altering disulphide bonds within the vaginal microenvironment during infection may promote long-term colonization in numerous ways. In addition, coronins constitute an evolutionary family of WD-repeat containing actin-binding proteins, which are cytoskeletal and membrane trafficking multifunctional regulators (Rybakin and Clemen, 2005). The amoeboid transformation of trichomonads upon cytoadherence requires cytoskeleton rearrangements, and thus the combined role for actin, coronins and HSPs as chaperones seems logical. Interestingly, actins and HSP70 are also secreted and delivered to the erythrocyte surface in *P. falciparum* (Vincensini *et al*., 2005) and to the parasite tegument in *S. mansoni* (Knudsen *et al*., 2005). Lastly, carbamate kinase, an enzyme involved in arginine metabolism is also secreted. Interestingly, this pathway is largely restricted to prokaryotes and has been found only in the two primitive eukaryotes *Giardia lamblia* (Edwards *et al*., 1992) and *T. vaginalis* (Linstead and Cranshaw, 1983). The carbamate kinase gene represents a complex genetic history that spans the evolutionary time period from the archaea to the primitive eukaryotes *Giardia* and *Trichomonas* (Minotto *et al*., 2000). Importantly, the absence of any human orthologues to this gene and its importance as a secreted metabolic enzyme in *T. vaginalis* makes carbamate kinase a primary target for novel drug design and/or a vaccine candidate with any limited side-effects in host.

We searched for known eukaryotic signal sequences among the secreted proteins using the SPdb, a signal peptide database (Choo *et al*., 2005). Given the high occurrence of lateral gene transfer in *T. vaginalis* (de Koning *et al*., 2000), we felt it appropriate to also search for a bacterial LPXTG motif or choline-binding repeats and/or secretory pathways associated with these secreted proteins. No signal peptide sequences were found. It is therefore conceivable that these metabolic enzymes are secreted from the parasites and are re-associated on the surface of the pathogen, thereby functioning as virulence factors by binding to host proteins (Pancholi and Chhatwal, 2003) and/or modifying host proteins to activate the signalling cascade to enable the progression of pathogenesis. Finally, it will be of interest whether the secreted metabolic enzymes in the trichomonad secreted protein family have alternative functions yet to be determined.

We now show that purified AP65 incubated with VECs increased expression of only IL-8 and not COX-2 and FN1, two genes upregulated by parasite contact with VECs (Kucknoor *et al*., 2005b). Moreover, the dramatic increased IL-8 synthesis confirms the earlier report by us (Kucknoor *et al*., 2005b) as well as work by others that patients have elevated levels of IL-8 in secretions (Shaio *et al*., 1994). It is possible that the extent of IL-8 found in secretions of patients (Shaio *et al*., 1994) is a function of the presentation to host cells of AP65, and this would have a pronounced effect on the extent of lymphocytic infiltration in vaginal secretions. A recent report showed episomal expression of the *H. pylori* CagA in gastric epithelial cells gave signalling for IL-8 induction (Kim *et al*., 2005). We therefore hypothesized that episomal AP65 within HeLa cells, that also accommodate *T. vaginalis* adherence, would likewise induce IL-8 expression and give results without interference from unknown factors thereby be a more direct means of

establishing a role for AP65 in host cell signalling. Importantly, during the course of this study, we verified the synthesis and cytoplasmic localization of AP65 within the transfected HeLa cells. Indeed, increased expression of both IL-8 and COX-2 was observed in these transfected cells (Fig. 5). These data provide evidence that different signalling pathways for expression of host genes result from presenting parasite proteins externally or within the host cells. Although requiring further experimental verification, that host cells during infection may sequester intracellularly parasite proteins is not inconceivable.

In our earlier report (Kucknoor *et al*., 2005b), we concluded that *T. vaginalis* organisms possessed some soluble factor to induce COX-2 gene expression in VECs. These data now indicate that compared with intracellular AP65 (Fig. 5), extracellular AP65 requires additional factors for signalling for optimal COX-2 gene expression (Fig. 1). We confirmed the presence of COX-2, which was readily visualized in the perinuclear region in *ap65*-transfected HeLa cells (Fig. 6). That AP65 affects the expression of cytokines or other growth factors that in turn induce COX-2 cannot be discounted. Further, COX-2 and DAD1, a member of Bcl2 gene family (Cory *et al*., 2003), which is also upregulated by trichomonads (Kucknoor *et al*., 2005b), are antiapoptotic. As such, these observations may be relevant in light of the non-self-limiting nature of trichomonosis, the significance of which may be related to cervical cancer (Yap *et al*., 1995; Zhang *et al*., 1995; Sayed el-Ahl *et al*., 2002) and prostate cancer (Sutcliffe *et al*., 2006). Therefore, these observations make evident the need to continue to characterize this family of secreted proteins of *T. vaginalis*.

Because parasite contact with VECs also upregulates expression of numerous trichomonad genes, including the prominent adhesin AP65 (Mundodi *et al*., 2004) and metabolic enzymes (Kucknoor *et al*., 2005a), it seemed reasonable to expect that the preparation of secreted proteins would be complex in total number of proteins. Given the fact that a functional trichomonad protein, like the AP65 adhesin (Arroyo *et al*., 1993; Garcia *et al*., 2003), induces VEC gene expression (Figs 4–6), it is also conceivable that other secreted proteins play important roles in the biology of this host–parasite interaction. This is the first attempt to characterize the secreted proteins of *T. vaginalis*. Much remains to be learned about the parasite virulence factors involved in symptomatology and pathogenesis among some women and men. Characterization of the secreted proteins may lead to an understanding of soluble factors in secretions of patients that play important roles in modulating the overall host response to trichomonosis, as evidenced by the role of AP65 in IL-8 and COX-2 upregulation of gene expression.

Experimental procedures

Parasites and host cells

Trichomonas vaginalis isolate T016 was grown in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum at 37°C (Diamond, 1957). Mid- to late-logarithmic phase trichomonads were used for all experiments. Immortalized MS-74 human VECs and HeLa epithelial cells in confluent monolayer cultures were prepared for adherence experiments, as before (Garcia *et al*., 2003). These epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) medium supplemented with 10% fetal bovine serum, at 37°C in presence of 5% CO_2.

Precipitation of extracellular proteins

Trophozoites (10^7 ml^{-1}) at ~18 h of growth were resuspended in RPMI medium (Invitrogen) and incubated for an additional 1 h at 37°C. Parasites were monitored throughout the growth and incubation periods to assure absence of cell lysis and viability using trypan blue exclusion assay and microscopic observations. Further, the protocol used here was from our earlier work, which carefully demonstrated that the soluble trichomonad proteins in cell-free culture supernatants was not due to lysis of parasites by various criteria (Alderete and Garza, 1984). Briefly, supernatant was clarified by gentle centrifugation at 500 *g* at 4°C, as before (Alderete and Garza, 1984). The resulting supernatant was filtered through a 0.22-μm-pore-size filter to remove insoluble debris. Filtered supernatant was immediately precipitated using 10% TCA (w/v) and incubated overnight at 4°C. The precipitate was centrifuged for 10 min at 10 000 *g*. The pellet was washed twice in 10 ml of acetone and air dried.

Two-dimensional sodium dodecylsulphate-polyacrylamide gel electrophoresis (2-D SDS-PAGE)

Isoelectric focusing gels and total protein samples were prepared using standard protocols (Hochstrasser *et al*., 1988). Protein samples were solubilized for 30 min at room temperature in 125 μl of rehydration buffer (9.8 M urea-100 mM DTT-4% CHAPS, and 0.2% Bio-lytes pI 3–10 in 0.001% bromophenol blue). For the resolution of protein samples, a Protean IEF Cell System (Bio-Rad) was used. One hundred μg of protein was separated on 11-cm immobilized pH gradient (IPG) strips (Bio-Rad) with a 3 to 10 linear pH gradient. IEF was conducted at 20°C for 12 h at a maximum voltage of 8000 V and maximum current of 50 μ amp/gel. The IPG strips were equilibrated twice with SDS equilibration buffer (3M urea, 2% SDS, 1% DTT and 10% glycerol in 125 mM Tris-HCl, pH 8.8). The strips were then transferred to precast 10% Tris-Glycine SDS-PAGE gels (Bio-Rad). Gels were always in triplicate, and all gels used samples from three independent batches of cell culture supernatants.

MALDI-TOF/MS analysis

Identification of protein spots after 2-D SDS-PAGE was accomplished by mass spectrometry. Protein spots were visualized by staining of gels with Bio-Safe Coomassie (Bio-Rad) and imaged with the GS-800 densitometer (Bio-Rad). Thirty-two protein spots were selected for analysis based on protein abundance and clear separation of individual spots. Selected spots were excised with

a biopsy needle *in situ* digestion with trypsin, according to standard protocols (Shevchenko *et al*., 1996). The resulting digests were analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an Applied Biosystems Voyager-DE STR (Framingham, MA) operated in reflector mode using delayed extraction. The peptide mass maps produced by MALDI-TOF/MS were searched against published databases using Mascot (Matrix Science) to provide information about the identity of the protein(s) in each spot. Sequence information and characterization of selected digests were accomplished with capillary-HPLC-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) on a Thermo Finnigan LCQ ion trap mass spectrometer coupled to a Michrom BioResources Paradigm MS4 micro HPLC by means of a home–built microspray interface. A data-dependent acquisition protocol was employed consisting of one survey scan followed by 4 collision-induced dissociation spectra. The CID spectra were searched primarily against the SWISSPROT followed by the TIGR database, which consisted of partially annotated *T. vaginalis* genome sequences (http://tigrblast.tigr.org/er-blast/index.cgi/project=tvg) using Mascot (Matrix Science; in-house license). A 95% confidence level threshold was used for Mascot protein scores (MALDI-TOF/MS) or peptide scores (ESI-MS/MS).

Isolation of total RNA

The MS-74 VECs were used for interaction experiments with trichomonads, as before (Garcia *et al*., 2003). Briefly, 1×10^5 VECs were seeded onto 6-well tissue culture plates and allowed to form a confluent monolayer. VECs were then washed with a medium mixture of DMEM : TYM (2:1, v/v) without serum. Secreted proteins from supernatant of trichomonal medium after growth was added to the MS-74 monolayer at different concentrations and incubated at 37°C for 2 h. In some experiments, purified AP65 protein obtained by affinity chromatography with monoclonal antibody (12G4 mAb) to AP65 was added to the monolayer at a concentration of 10 μg/well. Total RNA from MS-74 cells was isolated using Trizol reagent (Invitrogen).

Semiquantitative RT-PCR analysis of selected VEC genes

Total RNA (1 μg) was reverse transcribed with oligo (dT) primer using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. PCR amplification of cDNA was carried out using gene-specific primers (Kucknoor *et al*., 2005a). The gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR products were separated on 2% agarose gels with ethidium bromide. The band intensity was quantified using the Scion image beta program (http://www.scioncorp.com/pages/scion_image_windows.htm). PCR reactions were carried out at four different times to confirm reproducibility.

SDS-PAGE and immunoblotting

Equal amounts of TCA-precipitated secreted proteins and total *T. vaginalis* lysates were separated by SDS-PAGE on 10% acrylamide gels, and the proteins transferred onto nitrocellulose membranes (Bio-Rad). Nitrocellulose blots were blocked in 0.1% Tween 20 containing 5% BSA and probed with the 12G4 mAb to AP65 (Garcia *et al*., 2003), B512 mAb to α-tubulin (Sigma), and L64 mAb to an ~30 kDa cytoplasmic protein (Alderete *et al*., 1987). These antibodies served as controls for the various experiments. For example, mAb B512 to α-tubulin was to show membrane integrity. The 30 kDa cytoplasmic protein detected by mAb L64 reacts only with the cytosol fraction by immunoblotting and with the cytoplasm by fluorescence using permeabilized trichomonads. Thus, both these mAbs confirmed absence of lysis. AP65 in the preparation of secreted proteins was detected by 12G4 mAb. The blots were further incubated with secondary anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad). The blots were washed well and incubated in horseradish peroxidase substrate (Bio-Rad) to visualize proteins.

Episomal expression of AP65 in HeLa cells and visualization by microscopy

In order to express the *T. vaginalis ap65-1* gene in HeLa cells, the *ap65-1* open reading frame was amplified by PCR and cloned into the pC1Neo vector (Promega). The resulting plasmid termed pC1Neo-AP65 was purified using Qiagen Maxi kit. For RNA and protein analysis, HeLa cells were transfected in 6-well tissue culture plates. Transient transfection was carried out with 1 μg of purified plasmid using *Trans*IT-HeLaMONSTER transfection Kit (Mirus). For microscopic observation, HeLa cells were seeded on BD Falcon culture slides (Becton Dickinson) and grown to ~70% confluent monolayers for transfection, as above. After 24 h, the monolayer was washed and incubated in blocking buffer (PBS-5% BSA) followed by the addition of 1:1000 dilution of COX-2 mAb (Cayman Chemicals) and 12G4 mAb for 1 h. Fluoresceine isothiocyanate-conjugated anti-mouse IgG antibody was then added for 30 min at 4°C.

For detection of AP65 in VECs, MS74 cells were grown on BD Falcon culture slides. Purified AP65 or trichomonads was added to the monolayer (10:1 ratio) and incubated for 40 min. The wells on the slides were washed 5 times with PBS to remove the unbound protein as well as the bound trichomonads. The VECs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated with 12G4 mAb (1:1000) for one h followed by Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody (Invitrogen) for 30 min. The monolayer was washed with PBS prior to removing the chambers on the slides. Slides were processed for observation using an epifluorescence Olympus BX41 microscope.

Enzyme linked immunosorbant assay for the detection of IL-8

IL-8 from cell culture supernatant was analysed by ELISA using BD OptEIA Human IL-8 Kit II (BD Pharmingen), according to the instructions supplied by the manufacturer. Assay standardization and standard curves for IL-8 were carried out according to the kit instructions.

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