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Microreview

Enzymes on microbial pathogens and *Trichomonas vaginalis*: molecular mimicry and functional diversity

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Introduction

'Given the pragmatism of molecular evolution, multifunctionality of biological macromolecules may be more common than has been realized, and may affect rates of evolution and even medical therapies targeted at particular molecules with unanticipated secret identities.' Quote from Piatigorsky and Wistow (1991).

This Microreview will attempt to illustrate the importance of relatively new findings: those involving metabolic enzymes on the surface of numerous microbial pathogens. Seemingly remarkable at first, it is now accepted that microorganisms possess surface-associated metabolic enzymes (SAEs). The literature indicates that the SAEs may possess metabolic-enzymatic activity and also alternative functions. Although we believe that the evidence for alternative function(s) is strong, we realize that the physiological significance of SAEs has not yet been clearly established. The challenge to investigators, therefore, is the identification of the different function and elucidation of the *in vivo* role of the SAE. We will discuss SAEs on pathogens in terms of their functional diversity and contribution to survival of these microorganisms in complex host environments and their contribution to virulence. We have chosen to indicate that SAEs fall into the category of molecular mimicry, defined broadly, taking into account the immunological criteria as well as the more recent inclusion of adaptive and consequential mimicry, and readers are encouraged to read the excellent reviews on this subject (Oldstone, 1987; Damian, 1989; Hall, 1994). In the case of SAEs of microbial pathogens, their existence as host-like molecules may confer an immune evasion strategy (Damian, 1989) and, equally importantly, may mimic or subvert an equivalent host function. Immune (antibody) cross-reactivity with the host equivalent enzyme, such as enolase as discussed below (Fontan *et al*., 2000), may result in host cytopathology. A novel function for the host-like SAE would still be consistent with molecular mimicry.

Precedence for functional diversity of metabolic enzymes comes from one of the most remarkable and best-studied mammalian enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Here we will briefly review the functional diversity of GAPDH to set a knowledge baseline for those interested in this field and to illustrate that our overall knowledge with respect to the functional diversity of GAPDH and other enzymes is still in its infancy. What is learned from other experimental systems, including those examining the functional diversity of GAPDH within mammalian cells, will be of importance to the field of cellular microbiology. We will discuss the data showing the surface expression of candidate adhesins that resemble enzymes by the amitochondriate *Trichomonas vaginalis*, enzymes originally found compartmentalized in vacuoles called hydrogenosomes. Data suggesting an alternative role in cytoadherence for these SAEs and a role for iron in upregulation of gene expression as well as compartmentalization of the SAEs will be discussed. Recent fluorescence and immunoelectron microscopy data will be presented.

Investigators examining host–parasite interactions and understanding the molecular mechanisms of pathogenesis could not have predicted that microbial pathogens would have SAEs. What is exciting is the discovery of alternative functions for the SAEs, and a representative listing of microbial pathogens with SAEs and, where known, the function is presented in Table 1. These and other findings of the past decade on metabolic enzymes on microbial surfaces are consistent with the concepts of gene sharing and gene duplication with acquisition of new functions. These concepts were developed by investigators who studied the eye lens. The specialized refactory property of the lens is as a result of the production, in large concentrations, of soluble structural proteins

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Table 1. Examples of crystallines and surface-associated metabolic enzymes of microorganisms with acquisition of new functions.

Protein or function	Relationship or identity	Cell or organisms	Reference
Representative crystallins			
1. α	Small heat shock proteins	Vertebrates *Schistosoma mansoni* Ag p40	Piatigorsky and Wistow (1991)
2. δ	Argininosuccinate lyase (ASL)	Birds/reptiles	Piatigorsky and Wistow (1991)
3. ε	Lactate dehydrogenase-B (LDH-B)	Birds/reptiles	Piatigorsky and Wistow (1991)
4. ξ	Alcohol dehydrogenases	Mammals	Piatigorsky and Wistow (1991)
5. ρ	NADPH-dependent reductases	Frogs	Piatigorsky and Wistow (1991)
Microbial enzymes			
1. Binding to plasmin, fibronectin, lysozyme, myosin, and actin, and ADP-ribosylating enzyme	GAPDH	Group A streptococcus	Lottenberg, Broder *et al*. (1992);Pancholi and Fischetti (1992, 1993)
2. Binding to transferrin and plasmin	GAPDH	*Staphylococcus epidermidis*	Modun and Williams (1999); Modun *et al*. (2000)
3. Unknown	Neuramidase	*Streptococcus pneumoniae*	Camara, Boulnois *et al*. (1994)
4. Adhesin	Glucosyltransferase	*Streptococcus gordonii*	Vacca-Smith *et al*. (1994)
5. Plasmin receptor and antigen	α-Enolase	*Streptococcus pyogenes*	Pancholi and Fischetti, (1998); Fontan *et al*. (2000)
6. Cell growth dehydrogenase	NAD-depdendent glutamate	*Porphyromonas gingivalis*	Joe *et al*. (1994)
7. Antigen and binding to fibronectin	GAPDH	*Candida albicans*	Gil-Navarro *et al*. (1997); Gozalbo *et al*. (1998)
8. Flocculation	GAPDH	*Kluyveromyces marxianus*	Fernandes *et al*. (1993); Moreira *et al*. (2000)
9. 37 kDa immunogen	GAPDH	*Schistosoma mansoni*	Goudot-Crozel *et al*. (1989)
10. Adhesins AP65, AP51, and AP33	Malic enzyme (decarboxylating), and β-and α-SCS respectively	*Trichomonas vaginalis*	Alderete *et al*., (1995); O'Brien *et al*. (1996); Alderete *et al*. (1998); Engbring and Alderete, 1998a, b)

Table 2. Functional diversity of GAPDH in mammalian cells and microbial pathogens.

Function	Representative reference[a]
Mammalian cells	
1. Membrane fusion and transport	Morero *et al*. (1985); Hessler *et al*. (1998)
2. Nitric oxide	Dimmeler and Brune (1993); Brune and Lapetina (1995)
3. Apoptosis	Vartanian *et al*. (1997); Ishitani *et al*. (1998)
4. Neuronal disorders	Sawa *et al*. (1997); Wu *et al*. (1997); Ishitani *et al*. (1998)
5. Viral pathogenesis	De, Gupta *et al*. (1996); Carlile *et al*. (1998)
6. Prostate cancer	Scharief *et al*. (1994); Gong *et al*. (1996)
7. Endocytosis	Robbins *et al*. (1995)
8. Microtubule bundling	Kumagai and Sakai (1983)
9. Phosphotransferase/kinase	Duclos-Vallee *et al*. (1998); Engel *et al*. (1998)
10. Transcription/translational regulation	Morgenegg *et al*. (1986)
11. Nuclear RNA and tRNA export	Meyer-Siegler *et al*. (1991); Singh and Green (1993); Nagy and Rigby (1995); Zang *et al*. (1998)
12. DNA replication	Grosse *et al*. (1986); Baxi and Vishwanatha (1995)
13. DNA repair	Vollberg *et al*. (1987; Cool and Sirover (1989); Baxi and Vishwanatha (1995)
Microbial pathogens	
1. Plasmin-binding protein	Lottenberg *et al*. (1992); Pancholi and Fischetti (1992)
2. ADP-ribosylating enzyme	Pancholi and Fischetti (1993)
3. Phosphotransferase/kinase	Pancholi and Fischetti (1997)
4. Transferrin receptor	Modun and Williams (1999); Modun *et al*. (2000)
5. Binding protein for fibronectin, lysozyme, myosin, and actin	Pancholi and Fischetti (1992); Gil-Navarro *et al*. (1997); Gozalbo *et al*. (1998)
6. Flocculation	Fernandes *et al*. (1993); Moreira *et al*. (2000)
7. Immunogenic protein	Goudot-Crozel *et al*. (1989); Gil-Navarro *et al*. (1997)

a. A more extensive literature review is provided in references Sirover (1997, 1999).

referred to as crystallins (Wistow and Piatigorsky, 1987). It was surprising that these lens proteins with such unusual roles were not specialized structural proteins. These crystallins of the lens, a representative few listed in Table 1, turned out to be metabolic enzymes and/or stress proteins having important roles beyond those in metabolism (Wistow and Piatigorsky, 1987; Piatigorsky and Wistow, 1989, 1991; Wistow, 1993). Moreover, the crystallins were not even lens specific, as various different cell types and tissues synthesized the enzymes. These metabolic enzymes seemed to be directly recruited to new functions by modification of gene expression and not through immediate gene duplication. It was from the unique perspectives of these findings that the researchers developed the concepts of gene sharing and acquisition of new functions that precede gene duplication. Furthermore, since these findings, it has become accepted that proteins reside in compartments and/or locations other than those predicted from structure-function studies. The presence of proteins (SAEs) in unexpected locations is directly related to alternative and diverse functions for these previously characterized proteins (Smalheiser, 1996).

GAPDH

'As with many things in life, what is thought to be simple and relatively straightforward turns out to be quite complex and elaborate. In this regard, a number of studies, accelerating in the past decade (Meyer-Siegler *et al*., 1991), have indicated that GAPDH is not an uncomplicated, simple glycolytic protein.' Quote from the excellent review by Sirover (1999).

As indicated above, while this short review focuses on the significance of metabolic enzymes with functional diversity on the surface of microbial pathogens (Table 1), it is essential to the readers to review briefly what is known about one of the most studied mammalian cell metabolic enzymes, GAPDH. There are several reasons for doing this. There are some parallels between what is being found for GAPDH on microbial surfaces with what is known for mammalian cell GAPDH, but more importantly, a great deal of insight as to possible non-enzymatic activities of SAEs on microorganisms might be obtained. The diversity of functions that are now attributed to GAPDH is remarkable, and moreover, the indications are that much more remains to be discovered about the diverse activities of this molecule. Table 2 presents a current listing of the many known functions for GAPDH (Sirover, 1997, 1999). What began as a protein involved in a sequence of substrate-product enzymatic reactions in glycolysis has evolved to a complex picture in which GAPDH plays a role in both normal cellular functions and in cell pathology. About 15 distinct functions have been experimentally ascribed to GAPDH (Table 2), and it is noteworthy that each activity has been examined at the molecular level by numerous laboratories. While the underlying mechanisms for some of the known activities have yet to be delineated, it is clear that there are sequence-specific domains within GAPDH that govern a particular function. For example, nuclear RNA and tRNA export and the properties of DNA replication and repair

involve specific nucleotide sequences and GAPDH regions. Interactions between GAPDH with 3′ and 5′ end UTRs appear responsible for transcriptional and translational regulation. A role for GAPDH in neuronal disorders and apoptosis possibly involving the nitric oxide pathway as one mechanism highlights how a multifunctional protein may be involved in disease unique to a particular tissue. More recently, the idea that GAPDH is in an unknown way involved with Alzheimer's disease is reinforced by the findings of specific interactions between the enzyme and β-amyloid precursor protein (Schulze *et al*., 1993). GAPDH mRNA levels have been found to be greater in neoplastic tissue compared with benign prostate tissue, and it appears that both GAPDH mRNA and glycolytic activity are regulated, at least in part, by androgen exposure. This relationship will provide the impetus to examine the role of GAPDH in other cancers.

From a microbial pathogenesis perspective, the interaction of GAPDH with viral RNAs or viral proteins is of particular interest. That these associations may influence viral-mediated disease outcomes is intriguing. Equally importantly, the phosphotransferase and kinase activities of GAPDH that may involve signalling pathways is now important for group A streptococcal interactions with host cells, as will be highlighted in the section below. Interestingly, while it has been suggested recently that the functional diversity of GAPDH may be related to post-translational modification, such as through mono-ADP-ribosylation, this activity is an important attribute of the group A streptococcus surface-associated GAPDH (Pancholi and Fischetti, 1993).

Lastly, scientists studying SAEs of both mammalian and microbial cells are challenged by a need to understand how enzyme monomers and tetramers (in this case those of GAPDH) with identical structures are recruited to unexpected cell (either eukaryotic or prokaryotic) sites (Smalheiser, 1996). What are the signals that tell the cells exactly where subsets of identical enzyme monomers (at the nucleotide and amino acid sequence levels) are to be modified for targeting to a particular region (the surface in the case of microorganisms)? Where a monomer may have numerous functions, such as is the case for GAPDH of both mammalian cells and microbial pathogens (Table 2), what determines the particular function to be expressed? It is likely that knowledge gained from parallel studies of enzymes such as GAPDH of mammalian cells and SAEs of microorganisms will be mutually beneficial and synergistic.

Microbial pathogens

In summary, Table 1 lists some recent reports that describe metabolic enzymes on microbial surfaces (SAEs). Readers are encouraged to go to the original publications for details. The plasmin receptor for group A streptococci has been purified to homogeneity and identified as GAPDH by two groups (Lottenberg *et al*., 1992; Pancholi and Fischetti, 1993). The surface location of group A streptococcal GAPDH as well as other SAEs was confirmed on the basis of established cell fractionation and antibody immunoreactivity assays, such as immuno-electron microscopy. It was shown that GAPDH binds to fibronectin, lysozyme, myosin and actin, as well as having ADP-ribosylating and phosphotransferase activity (Pancholi and Fischetti, 1997a). The ability of GAPDH to readily associate with different substrates was demonstrated on the basis of binding of radioiodinated SAE with immobilized lysozyme, myosin and its globular domain (heavy meromyosin), and actin. Indicative of specific interactions, no similar binding was seen with the α-helical domain of myosin (light meromysin), the S-2 fragment of heavy meromyosin, and the streptococcal M protein. Moreover, immobilized streptococcal GAPDH bound fibronectin. It is not known, however, whether the association with fibronectin, as seen in a ligand-blot assay, contributes solely or in a co-operative fashion with other known fibronectin-binding proteins of this microorganism (Hanski and Caparon, 1992; Hanski *et al*., 1992, 1996) to host colonization. Interestingly, a surface-associated α-enolase of group A streptococci has been identified using approaches and criteria used for GAPDH (Pancholi and Fischetti, 1998). Consistent with the known plasmin-binding activity of surface α-enolase of mammalian cells, this SAE also has a strong plasmin-binding property with higher affinity compared with GAPDH. The presence of two types of plasmin-binding proteins with different affinities has been reported for group G streptococci (Ullberg *et al*., 1992), although the identity of the receptor is unknown. The true role of these SAEs in group A streptococcal pathogenesis awaits clarification.

Immuno-electron microscopy has shown that a neuraminidase is located on the surface of *Streptococcus pneumoniae* (Camara *et al*., 1994). While a distinct function has not been ascribed to this surface protein, it is of interest that this gene contains two possible start codons, which may permit synthesis of two forms of the neuraminidase from the single gene. For one form, a putative signal sequence in the N terminus, similar to the consensus for signal peptides of Gram-positive bacteria, would allow surface sequestration. It is known that *Actinomyces viscosus* neuraminidase has a leader peptide (Yeung, 1993) and that neuraminidase of *Bacteroides fragilis* is surface exposed (Guzman *et al*., 1990). Activity of the surface neuraminidase of *B. fragilis* appears to be related to bacterial attachment to epithelial cells (Guzman *et al*., 1990). *Streptococcus gordonii* possesses a surface-associated glucosyltransferase as an adhesin (Vacca-Smith *et al*., 1994), showing the diversity of

mechanisms employed by pathogens for host colonization. A surface protein (AP153) of *S. gordonii* also found in abundance in spent culture medium was identified as a candidate adhesin. Antibodies to AP153 inhibited bacterial attachment to cells in monolayers. This protein possessed glucosyltransferase activity.

The growing numbers of reports showing different microbial pathogens with surface-associated and functionally distinct GAPDH strengthen the observations from mammalian cells on the diversity of this protein. (1) GAPDH is also a potent antigen on the surface of the fluke *Schistosoma mansoni* (Goudot-Crozel *et al*., 1989). (2) It is a transferrin-binding protein (Tpn) for *Staphylococcus epidermidis* (Modun and Williams, 1999; Modun *et al*., 2000), and Tpn retains its glycolytic activity on the surface of intact staphylococci and in cell wall preparations. Perhaps not surprisingly, Tpn also has the capacity to bind plasmin, which retains its enzymatic activity on the staphylococcal surface (Modun and Williams, 1999). (3) There is strong evidence that GAPDH is an adhesin of *Candida albicans*, by binding to fibronectin and laminin (Gil-Navarro *et al*., 1997; Gozalbo *et al*., 1998; Staab *et al*., 1999). The surface location was apparent with immunoelectron microscopy using specific antibody, and both antibody and purified protein markedly reduced Candida associations with fibronectin and laminin. Not unexpectedly, the purified surface GAPDH bound to immobilized fibronectin and laminin in a ligand-blot assay. This binding of fibronectin by GAPDH is distinct from that which occurs through Candida surface integrin-like receptors (DeMuri and Hostetter, 1996; Hostetter, 1996, 1999). (4) Moreover, a multigene family of GAPDH exists for *Kluyveromyces marxianus*, and only one member of this family, p37 or GAP1, resides on the cell wall and mediates flocculation (Fernandes *et al*., 1992, 1993, 1995; Moreira, *et al*., 2000). Interestingly, regulation of expression of GAP1 may result from carbon sources different from those important for the other GAPDH genes. Genetic studies using a non-flocculating *Saccharomyces cerevisiae* transformed with GAP1 has resulted in a flocculation phenotype.

Additionally, the separate functions for GAPDH on group A streptococcus (Table 1) clearly illustrate that it is conceivable that this enzyme may contribute significantly to virulence and pathogenesis. For example, it was found that group A streptococcus surface GAPDH, also called streptococcal surface dehydrogenase (SDH), activates protein tyrosine kinase as well as protein kinase C of the pharyngeal cell (Pancholi and Fischetti, 1997b). These enzymes in turn tyrosine phosphorylate host cell proteins, showing the complex host response(s) after this initial interaction. Inhibition of the protein kinases significantly abrogated streptococcal invasion of the pharyngeal cells. The data suggest strongly that SDH plays an important role in communication between bacterium and the host cell, and such understanding of the consequences of this communication will probably be key to appreciating the pathogenesis of streptococcal infection.

While the *in vivo* relevance of some of the diverse functions of the SAEs may be questioned, the existence of similar, if not identical, host enzymes, some of which are surface expressed, may lead to cytopathology due to production of autoantibody (Oldstone, 1987; Damian, 1989), and of interest will be if host antibody responses, such as that seen for the GAPDH in patients with schistosomiasis (Goudot-Crozel *et al*., 1989) will lead to immune cross-reactions with host enzymes. Biliary cirrhosis, a chronic cholestatic liver disease, appears to result from auto-antibody to α-enolase surface expressed on mammalian cells (Akisawa *et al*., 1997), establishing precedence for anti-α-enolase antibody resulting from microbial surface expression of this host-like enzyme (Pancholi and Fischetti, 1998). Furthermore, *C. albicans* has a cell wall-associated and secreted α-enolase (Sundstrom and Aliaga, 1994; Angiolella *et al*., 1996), and patients with invasive candidiasis make antibody to α-enolase. It is not surprising, therefore, that patients with streptococcal pharyngitis, but not healthy control subjects, produce antibodies to streptococcal surface α-enolase (Fontan *et al*., 2000). The anti-streptococcal α-enolase antibodies react with the α-enolase on the mammalian cell surface, reinforcing the idea of post-streptococcal sequelae due to microbial SAEs (Fontan *et al*., 2000). Yet another example is the presence of anti-Tpn (anti-GAPDH) antibodies from patients suffering from staphylococcal peritonitis (Modun *et al*., 2000).

If the true role of the SAE can be established *in vivo* through genetic approaches, then a possible strategy for interference of infection can be envisioned. One area of interest will be the development of novel drugs or immune-based reagents. Indeed, abortion of SAE function may prevent or limit infection and disease outcomes. However, use of drugs affecting SAEs must now also take into account possibly concomitant unintended consequences to the host. For example, drugs targeting microbial GAPDH may lead to the unintentional disruption of a cellular function essential to the well being of normal host cells and tissues. This may occur where related host GAPDH (or other enzymes) with so many alternative functions may be affected by the specific drug.

In conclusion, it may be generally agreed that genetic approaches on the microorganisms with SAEs must be more fully utilized to prove a role for SAEs in virulence. However, the fact that these are enzymes may make it difficult or impossible to generate viable knockout mutants, even if the SAEs exist as multigene families as has been found for GAPDH of *K. marxianus* (Fernandes *et al*., 1995) and the candidate adhesins of *T. vaginalis*

discussed below. Alternatively, disruption of gene expression and/or function of SAEs may alter cellular functions that may bias the results. These studies nonetheless highlight both our lack of knowledge of the mechanisms of pathogenesis and of the complexity of the host-microbial/parasite interactions.

Trichomonas vaginalis

Background

Trichomonas vaginalis is a protozoan parasite responsible for the vaginitis trichomonosis (Kassai *et al*., 1988), the number one, non-viral sexually transmitted infection world-wide (WHO, 1995; Center for Disease Control and Prevention, 1996). This urogenital mucosal parasite recognizes and binds to mucin as a first step in colonization (Lehker and Sweeney, 1999). The trophozoites then cytoadhere squamous vaginal epithelial cells (VECs) but not to intermediate epithelial and parabasal cells (Alderete, 1988), suggesting host cell tropism. Specificity in cell targeting is further indicated by our finding that trichomonads were refractory to binding of fibroblasts (Alderete and Garza, 1985) and to primary cultures of human urinary tract epithelial cells (L. Chang and J. F. Alderete, unpublished data), providing further evidence of *in vivo* host cell specificity. VEC cytoadherence by trichomonads is complex, as evidenced by the signalling for dramatic morphological transformation (Alderete *et al*., 1988) that occurs within minutes after attachment to VECs. There is specificity in the signalling process as HeLa cells give no similar signal.

Receptor–ligand-type interactions appear involved between trichomonads and epithelial cells (Alderete *et al*., 1988; Arroyo *et al*., 1992, 1993). The surface structures on VECs recognized by *T. vaginalis* organisms are unknown. Four-trichomonad surface proteins (referred to as AP65, AP51, AP33 and AP23 based on electrophoretic mobilities and collectively as AP proteins) were identified as mediating cytoadherence. Numerous established criteria (Beachey *et al*., 1988) to show that the AP proteins were candidate adhesins were fulfilled (Arroyo *et al*., 1992; Alderete *et al*., 1995a; Engbring *et al*., 1996; Alderete, 1999a). (1) The proteins reside on the trichomonal surface as evidenced by surface radioiodination and indirect immunofluorescence experiments. (2) There is a direct relationship between amounts of surface AP proteins and levels of cytoadherence. (3) Removal of the surface AP proteins by trypsinization decreased cytoadherence. (4) Regeneration of adhesin synthesis and surface placement by incubation of treated parasites in growth medium restored the adherence phenotype. (5) Treatment of host cells with purified adhesins inhibited attachment by trichomonads in a concentration-dependent fashion. (6) Not unexpectedly, pre-treatment of organisms with different amounts of antibody to each of the AP proteins inhibited cytoadherence in a concentration-dependent manner. (7) Synthesis of the four adhesins was co-ordinately upregulated by binding to epithelial cells (Arroyo *et al*., 1993) and by iron (Arroyo *et al*., 1992). Growth in iron-restricted medium gave parasites with both decreased amounts of detectable AP proteins and low levels of cytoadherence. (8) Recombinant AP proteins compete with binding of the natural trichomonad adhesin proteins to host cells. (9) A receptor-binding epitope identified on AP33 competed with the natural adhesin for binding to host cells. Of special interest was that the receptor-binding epitope peptide, but not a control peptide, itself inhibited cytoadherence by live *T. vaginalis* organisms to HeLa cells (Engbring and Alderete, 1998a, b).

In support of the observations on the identity and role of the AP proteins, another group has found AP65 on the surface of *T. vaginalis* acting as an adhesive protein for erythrocytes (Rappelli *et al*., 1995) and have reported on the extracellular release of decarboxylating malic enzyme during growth and multiplication (Addis *et al*., 1997; Rappelli *et al*., 1998). More recently, this group has shown that, under their experimental conditions, the four AP proteins from detergent extracts bind to different cells in monolayer cultures and to *Mycoplasma hominus* (Addis *et al*., 2000). Unfortunately, the different cells and organism were not treated with glycine after fixation to avoid non-specific associations with these and other trichomonad proteins. Additionally, no cytoadherence assays were performed to correlate their observations with bona fide levels of associations with host cells and bacteria with amounts of the four proteins. The finding that *T. vaginalis* adhesins bind erythrocytes and bacteria may not be surprising given the extensive literature on the ingestion of both of these, presumably as sources of nutrients. Nonetheless, a likely interpretation of their results is that a structure or structures common to each of the cells and possibly on *M. hominis* is being recognized by the AP proteins. Differences in the content of these structures among the cells and bacterium may also explain why the amounts of the individual proteins are variable, unlike what has been reported for HeLa cells, for example (Arroyo *et al*., 1992). Alternatively, it is possible that the AP proteins are promiscuous in recognition of different surface structures, as was recently found for the erythrocyte membrane protein 1 of *Plasmodium falciparum* (Chen *et al*., 2000). Regardless of the mechanism involved, this group has shown the uptake by *T. vaginalis* of *M. hominus* (Rappelli *et al*., 1998), indicating a role for the AP proteins in these possibly specific associations. Furthermore, it is noteworthy that what was

not questioned by this group was the role of these AP proteins in *T. vaginalis* recognition and binding to VECs.

More recent work reveals that three of the four adhesins studied to date are each members of multigene families (Alderete *et al*., 1995b, 1998; Arroyo *et al*., 1995; Engbring and Alderete, 1998b). Importantly, sequence analyses at both the nucleotide and amino acid levels revealed structural molecular mimicry of adhesins with known metabolic enzymes (Engbring *et al*., 1996). Three of the adhesins have sequence identity with decarboxylating malic enzyme (AP65) and the α-and β-subunits of succinyl coenzyme synthetase (SCS) (AP33 and AP51 respectively), enzymes compartmentalized to vacuoles called hydrogenosomes (Müller, 1997). Hydrogenosomes are double membrane-bound organelles involved in fermentative oxidation of pyruvate derived from glycolysis (Müller, 1997). Analysis of the receptor-binding epitope for the adhesin AP33 identified the 24-amino-acid binding domain with the ability to inhibit parasite associations with host cells (Engbring and Alderete, 1998a). It was noteworthy that purified, commercially available enzymes with identity to the adhesins were incapable of inhibiting binding of the recombinant and natural adhesins to host cell surfaces and preventing trichomonal cytoadherence (Alderete *et al*., 1995a, 1998; Engbring *et al*., 1996; Engbring and Alderete, 1998a, b). It is also noteworthy that differences exist in the 3′ and 5′ end UTRs for each of the family members. Found within the 3′ end of some of the genes were AT-rich destabilizing sequences that may be important for transcript half-life possibly determining expression of hydrogenosomal vs. surface targeting of the proteins. Finally, the N terminus of the adhesins has a short signal sequence (Lahti *et al*., 1992) that may play a role in targeting to hydrogenosomes. However, it is interesting that a similar N terminus sequence is found for other trichomonad surface proteins, such as the P270 immunogen (Musatovova and Alderete, 1998 and unpublished observations) that undergoes the property of phenotypic variation for dsRNA virus-infected *T. vaginalis* isolates (Alderete *et al*., 1986a, 1987; Wang *et al*., 1987), showing that this signal sequence may play a role in targeting to non-hydrogenosomal membranes. As will be pointed out below, the AP proteins in response to iron are found in hydrogenosomes, on the surface and in other cellular compartments. This indicates that there exists other protein sequence-specific motifs or factors mediating translocation of the AP proteins within trichomonads. This may not be surprising given that many of the SAEs described in this review (Table 1) have no obvious leader sequence or membrane-targeting region.

Surface location of adhesins

As the surface location of these cytadhesins is a requirement for function, it was important to visually localize the trichomonad adhesins by fluorescence (Fig. 1) and immuno-electron microscopy (IEM) (Fig. 2). Fresh clinical isolates of *T. vaginalis* were grown under iron-replete and iron-limiting medium conditions prior to examination with both rabbit antisera and monoclonal antibody (mAb) to the adhesins, which have been characterized before (Alderete, *et al*., 1986b, 1988, 1995b, 1998, 1999b; Wang *et al*., 1987; Lehker *et al*., 1991; Arroyo *et al*., 1992; Engbring and Alderete, 1998a, b). It is noteworthy that both antisera and mAb gave identical immunoblot reactivities after two-dimensional SDS–PAGE (Engbring and Alderete, 1998a).

Figure 1 shows that little surface expression of AP65 was evident for organisms grown under low iron, a condition known to downregulate expression of adhesin synthesis (Alderete *et al*., 1995a; O'Brien *et al*., 1996), as seen for both permeabilized (Fig. 1A) and non-permeabilized cells (Fig. 1B). On the other hand, a greater intensity of surface fluorescence was evident for the non-permeabilized high-iron trichomonads (Fig. 1C). Further and as expected, the surface expression of P270, the phenotypically varying immunogen mentioned above, was readily detected for non-permeabilized, low-iron trichomonads, consistent with an earlier report (Alderete, 1999a). These results are consistent with the summary and published reports given above and reaffirm the surface expression of the SAEs characterized as trichomonad adhesins.

Interestingly, IEM performed on high-iron-grown organisms comparing rabbit antiserum and mAb with AP65 revealed major differences in localization. Rabbit antiserum to AP65 readily detected adhesin in the Golgi, endoplasmic reticulum, hydrogenosomes (dark contrast vacuoles), other vacuoles, cell surface and the trichomonad flagella. In contrast, the immunoreactivity with mAb was less intense and revealed only adhesin protein within hydrogenosomes (Fig. 2A). Equally noteworthy, low-iron organisms had overall weaker immunoreactivity for mAb and also for rabbit antiserum, reinforcing past findings that showed decreased synthesis and surface expression of adhesins in low-iron growth conditions (Lehker *et al*., 1991). These results reaffirm the surface location for the adhesins and illustrate that iron not only regulates adhesin synthesis but is also involved in modulating compartmentalization of adhesins. Furthermore and importantly, the data show selective detection by rabbit antiserum, but not mAb, of adhesins in the various compartments. Finally, these data illustrate that, depending upon the environmental conditions in which trichomonads are cultivated and the antibody reagents used, data can be generated that are conflicting from published reports (Brugerolle *et al*., 2000). This work will ultimately contribute to our understanding of structure-function properties for these

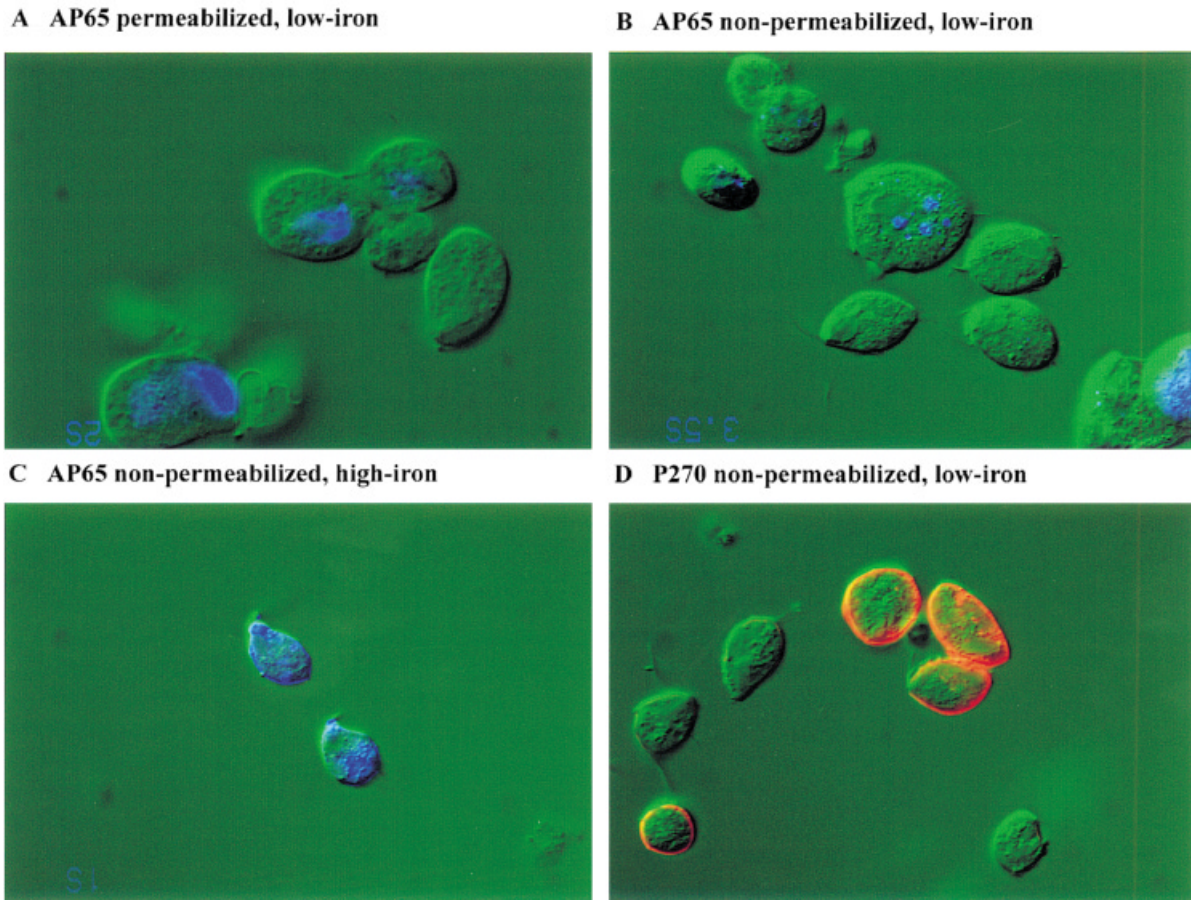

Fig. 1. Immunofluorescence showing surface expression of adhesin AP65 (A, B and C) and of the phenotypically varying P270 (D) of *Trichomonas vaginalis* isolate 347 organisms used before (Wang *et al*., 1987; Khoshnan and Alderete, 1993). Parasites were grown in either iron-limiting (A, B and D) or iron-replete (C) medium. Immunoreactivity of trichomonads was performed with anti-AP65 adhesin serum (Arroyo *et al*., 1992; O'Brien *et al*., 1996) and C20A3 mAb reactive with P270 (Alderete *et al*., 1986). Trichomonads were either permeabilized (cytoplasmic and surface labelling) or non-permeabilized (surface labelling) prior to incubation with antibodies. This isolate is designated Type II as defined by infection with a dsRNA virus (Alderete *et al*., 1987; Wang *et al*., 1987; Khoshnan and Alderete, 1993). Parasites at late logarithmic phase of growth were first washed in warm PHEM buffer (50 mM $MgCl_2$, 70 mM KCl, 10 mM EGTA, 20 mM HEPES, 60 mM Pipes, pH 6.8) and then allowed to adhere to cover slips previously coated with poly L-lysine. Thereafter, they were fixed with 4% paraformaldehyde in 100 mM cacodylate buffer, pH 7.2. Alternatively, trichomonads were fixed and permeabilized with 70% ethanol at −20°C or with 0.2% Triton X-100. Fixed organisms were quenched using 50 mM NH_4Cl with 3% BSA. Afterwards, the cells were washed in PBS containing 2% BSA, pH 8.0, for 30 min. Parasites were then incubated for 3 h with antibodies, as indicated. After washing in PBS, the samples were treated for 1 h with fluoroisothiocyanate-labelled anti-rabbit or mouse antibody for visualization of bound antibody. The organisms were finally washed in PBS and mounted onto slides using N-propyl-gallate. Cells were examined with an Axiophot 2 lense in a Zeiss fluorescent microscope.

functional proteins and of the role of iron in determining the compartmentalization of the SAEs within *T. vaginalis*.

General conclusion and comments

In retrospect, it is not surprising to find metabolic enzymes localized on surfaces of microbial pathogens and to have the SAEs display functional diversity as they do within mammalian cells. The findings of SAEs with newly acquired function for microorganisms provides us with long sought-after insight into how these small creatures with limited genomes survive in the complex host environments. Whether on mammalian cells or microorganisms, the range of function that some of these enzymes display is remarkable and may result from the multitude of three-dimensional structures, each of which is governed by the particular environment at the location. Within the accumulated evidence on the diversity of function for GAPDH, it may not be surprising to learn that enzymes function as molecular mimetics to host proteins, for example surface-associated GAPDH on group A streptococci and *C. albicans*. It is intriguing that SAEs may be virulence determinants, as evidenced by receptors for host proteins (plasminogen) and adhesins (functional diversity) respectively. Furthermore, because of their structural similarity to host molecules, they may

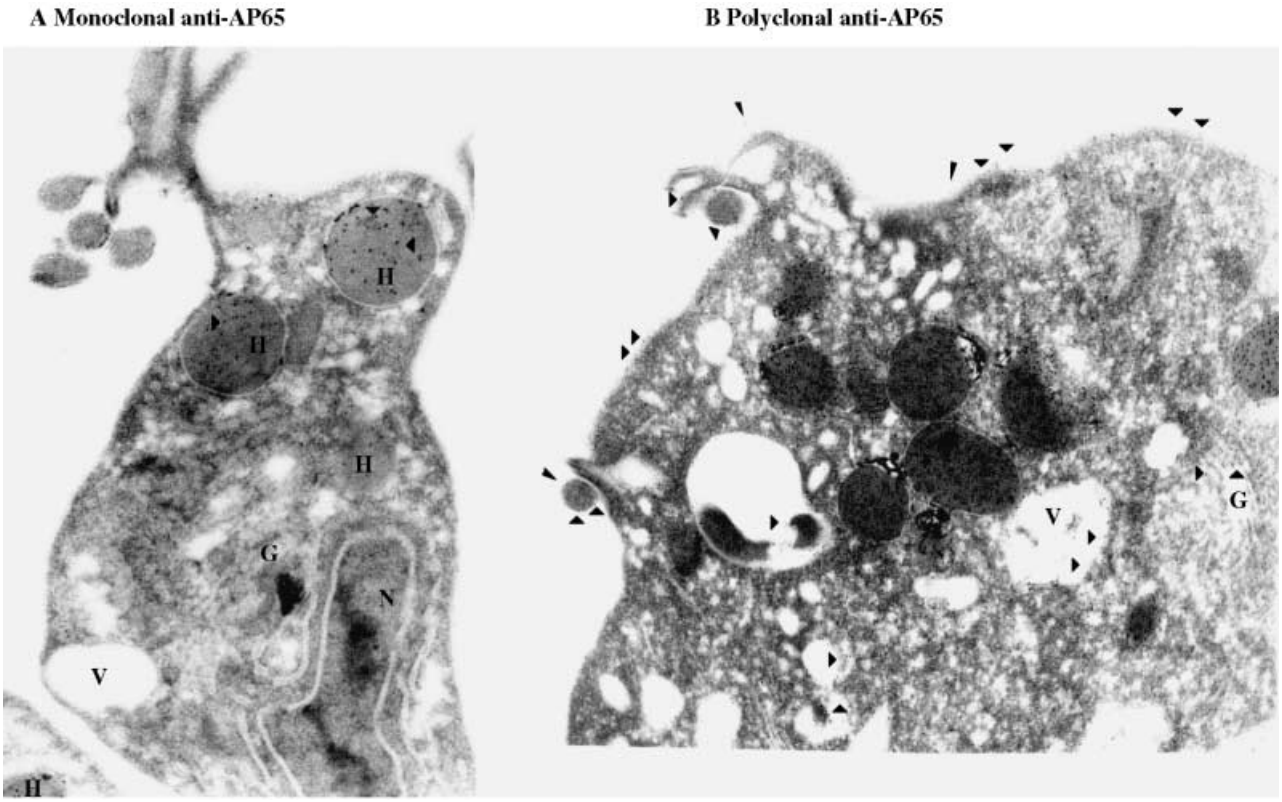

Fig. 2. Immuno-electron microscopy showing differential detection of AP65 adhesin proteins with mouse mAb F11 (A) in comparison to rabbit antiserum (B) to the AP65 adhesin. For these experiments, trichomonads of *T. vaginalis* isolate T068-II (Arroyo *et al*., 1992, 1993; Engbring *et al*., 1996; O'Brien *et al*., 1996) were washed three times with PHEM buffer at 37°C as above in Fig. 1. Organisms were then fixed overnight in a mixture of 4% paraformaldehyde, 0.4% glutaraldehyde, 1% picric acid, and 100 mM cacodylate buffer, pH 7.2 followed by dehydration in ethanol and infusion in Unicryl (BB International). For immunolabelling, thin sections were first incubated in PBS containing 3% albumin and quenched in 50 mM NH_4Cl for 30 min. The trichomonads were then incubated with mAb or rabbit antiserum to AP65 for 3 h. After several washes in PBS-1% BSA, sections were incubated in the presence of 10 nm gold-labelled goat anti-rabbit IgG antibodies (BB International). After several additional washings, sections were stained with uranyl acetate and lead citrate prior to observing in a JEOL 1210 electron microscope. Controls included omission of the primary antibody followed by all treatments. Prior work has demonstrated the absence of any immunoreactivity with control hybridoma supernatant and normal rabbit serum (Arroyo *et al*., 1992, 1993, 1995; Alderete, O'Brien *et al*., 1995, 1998; O'Brien *et al*., 1996; Alderete, 1999).

contribute to pathogen survival through molecular mimicry (Oldstone, 1987; Damian, 1989; Hall, 1994). As such, SAEs must be examined in detail within the general area of microbial pathogenesis research.

For *T. vaginalis*, the fact that each of the three adhesins studied to date are members of multigene families presents future challenges in delineating whether only some or all genes encode for the enzymatic and adhesive functions. Important subtle differences exist for the individual adhesin gene members. As mentioned above, the presence of AT-rich destabilizing elements on 3′ end UTRs of some, not all, the genes (Alderete *et al*., 1995b, 1998), while influencing transcript half-life, may also permit for compartmentalization of adhesins produced in greater quantities by stable transcripts, such as that seen in iron-replete medium. The fact that variations in iron growth conditions influences the distribution of adhesins within the parasites further illustrates that other factors are involved in compartmentalization or sequestration (chaperoning) of molecules to alternative locations other than vacuoles (hydrosomes) for metabolic function. Here, too, it will be necessary to identify those protein–amino acid sequences critical for site localization as well as non-enzymatic (adhesive) function. Finally, these results make clear the importance of utilizing multiple antibody reagents to the same protein for making conclusions about the true role(s) of SAEs in microbial pathogens. Ultimately, however, gene disruption experiments targeting entire families and individual members of the multigene families will be needed to prove the adhesive function and relative contribution of each of the AP proteins to host cytoadherence.

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