Encystation of *Giardia lamblia* Leads to Expression of Antigens Recognized by Antibodies against Conserved Heat Shock Proteins

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During in vitro encystation, *Giardia lamblia* expresses several stage-specific proteins which are recognized in immunoblots by antisera raised against antigens from three different pathogens. The antigens belong to two families of conserved stress proteins: (i) HSP60 purified from *Legionella pneumophila* and recombinant HSP60 from *Mycobacterium bovis BCG* and (ii) recombinant HSP70 from *Plasmodium falciparum*.

The cyst form of *Giardia lamblia* allows it to survive outside the host and to initiate new infection (23). We have induced cultured *G. lamblia* trophozoites to encyst in vitro and have shown that differentiation results in expression of a number of new antigens not observed in control trophozoites (21). At least some of these antigens are transported to the developing cyst wall by novel encystation-specific secretory vesicles (ESV) (21, 22). Although it is likely that these antigens are important in encystation, virtually nothing is known of their structure or function. Slightly alkaline pH (7.8) and bile induce expression of cyst antigens (11, 13, 14), formation of ESV (21), and development of water-resistant, biologically active cysts (4). These conditions, which mimic the small intestinal milieu, may also be cellular stress factors for the parasite. Alkaline pH induces a stress response in *Escherichia coli* (28), and bile salts, which are potent detergents, are toxic to most cells (6). Therefore, in the present studies, we tested the proteins expressed during differentiation of *G. lamblia* for reactivity with antibodies against certain highly conserved stress or heat shock proteins (HSP).

Recently, a number of immune-reactive proteins were found to correspond to the conserved HSP60 family of *Mycobacterium* species (26, 27) on the basis of sequence homology and antigenicity. These proteins include the 60- to 65-kDa common antigen previously described in gram-negative bacteria (15) and the GroEL gene product of *E. coli* originally defined by its role in bacteriophage growth (27). In its native state, bacterial HSP60 exists as a high-*M*, complex with HSP10, the product of the groES gene (29, 32). In eukaryotic cells, HSP60-like proteins associate with mitochondria or chloroplasts. The HSP60 proteins play roles in protein folding, assembly, and translocation across membranes (32).

If not otherwise specified, *G. lamblia* WB, clone C6, was used. Clone 1F, which encysts efficiently but differs from C6 in the type of cysteine-rich variable surface protein it expresses, was isolated from C6 by selection (19) with antibody (12) and complement. A6 refers to a subline of strain WB (from T. Nash) which encysts poorly under our conditions (unpublished data) and expresses a distinct variable surface protein (1). C6 and A6 have been cultivated separately for >8 years.

Parasite growth (7) and encystation (11) have already been described. Briefly, control nonencysting trophozoites were grown to confluence (48 to 72 h) in modified TYI-S-33 medium with bovine bile (16). Pre-encystation monolayers were grown in TYI-S-33 without bile at pH 7.1 (11). Encystation was initiated by refeeding these monolayers with TYI-S-33 medium supplemented with porcine bile (250 µg/ml) and lactic acid (5 mM) at pH 7.8. At 48 h of incubation, when >80% of trophozoites were expressing ESV and cyst antigens (9), total cultures were harvested by chilling and centrifugation, unless otherwise specified.

The heat shock treatment was performed by incubating trophozoites in growth medium at 41°C for 30 min and then allowing them to recover for 30 min at 37°C (18). Parasite antigens were separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17) and transferred to nitrocellulose (30) by standard methods. Reactions with rabbit anti-HSP antibodies were detected with alkaline phosphatase-conjugated protein A (Zymed, San Francisco, Calif.).

The Western blot (immunoblot) in Fig. 1 shows that rabbit antiserum against the HSP60 protein purified from *Legionella pneumophila* (20) reacted strongly with a broad protein band at 27 kDa which was detected only in encysting *Giardia* organisms. The 27-kDa *Giardia* protein was observed from ∼8 to 66 h of encystation (data not shown) but was not detected in heat-shocked or control, nonencysting trophozoites. This antiserum also detected proteins of ∼65 and 100 kDa, but only in heat-shocked cells. Preimmune serum from the same rabbit showed no reactivity (Fig. 1). Moreover, anti-*Legionella* HSP60 was monospecific in that it reacted in Western blots with a single protein in the HSP60 family in every *Legionella* species tested (20).

We next asked whether expression of p27 is limited to encysting cells or is induced by exposure to alkaline pH and bile, regardless of the ability to encyst. Therefore, we compared the reactivities of antigens prepared from three sublines of strain WB after 48 h of exposure to bile and lactic acid at pH 7.8. In contrast to sublines C6 and 1F, which encyst efficiently, subline A6 does not encyst well under
these conditions, forming <5% water-resistant cysts, compared with 1F and C6. Subline A6 also fails to efficiently express antigens recognized by antisera to the cyst wall or to form ESV when exposed to these stimuli (unpublished data).

The absence of p27 in subline A6 (Fig. 2) shows that its expression is linked to encystation. Expression of p27 by subline 1F shows that it is not limited to cells with the TSA417 cysteine-rich surface protein. Solely on the basis of its molecular weight, p27 may be not a homolog of GroEL but simply a cross-reactive protein. It appears more likely that the 65-kDa protein recognized by the anti-HSP60 serum in heat-shocked trophozoites is a GroEL homolog.

To characterize G. lamblia proteins expressed during encystation further, we tested their reactivity to rabbit antisera raised against Mycobacterium bovis BCG HSP60 purified from an E. coli recombinant (25, 26). This antibody reacted with a protein of ~57 kDa in encysting but not in heat-shocked or nonencysting control trophozoites (Fig. 3). p57 appears to differ from the 65-kDa protein of heat-shocked G. lamblia which was recognized by the Legionella antibody.

It should be noted that HSP60 analogs range from ~57 to 68 kDa in various microorganisms and have both conserved and unique epitopes (27). In addition, some bacteria, such as M. tuberculosis, encode two HSP60 analogs (25). Interestingly, the Legionella HSP60 antisera appears to have broader cross-reactivity than that raised against M. bovis BCG. For example, the Legionella antibody detects both GroEL homologs expressed by M. tuberculosis, while the M. bovis BCG antibody detects only one (25).

The HSP70 group of stress proteins is a complex, conserved family of constitutive and regulated proteins functionally distinct from the HSP60 group. Recently, an HSP70 protein has been shown to be expressed during differentia-
up regulated by bile starvation and have found that bile acids bind to a cytosolic protein (5a). Similarly, we had shown earlier that bile starvation stimulated encystation upon subsequent addition of bile (11).

Lindley et al. recently demonstrated a classic heat shock response in G. lamblia (18). Synthesis of [35S]methionine-labeled proteins of 30, 70, 83, and 100 kDa was enhanced at 43°C and also in response to other physical or chemical stresses. Our attempt to use the more physiological temperature of 41°C may not have induced the full heat shock response. Furthermore, the antibodies we used may not have detected the full heat shock response. Therefore, the relationships between these sets of findings are not clear.

In other studies, Aggarwal et al. (2) proposed that the "transformation of G. lamblia from the trophic to the cystic form may be induced by a heat shock response." They used a cloned Drosophila hsp70 promoter DNA fragment to isolate a G. lamblia gene with a possible heat shock promoter. Although expression of the gene increased over time at 42°C, the sequence of the open reading frame revealed no homology with Drosophila HSP70.

In most eukaryotic cells, proteins of the HSP60 family tend to be associated with mitochondria or chloroplasts (5), organelles not found in G. lamblia. The HSP60 group of proteins participates in the folding and assembly of polypeptides (24).

The HSP70 family includes constitutively synthesized and stress-regulated proteins with various cellular locations (24). HSP70-related proteins are associated with clathrin-coated pits (10). These HSP generally function as molecular chaperones, binding to newly synthesized or damaged proteins in their unfolded form and permitting their importation into membranous cellular compartments, such as mitochondria, chloroplasts, endoplasmic reticulum, and lysosomes. In previous studies, we found that encystation of G. lamblia entails assembly of a new class of membrane-bound organelles, large secretory vesicles, or ESV, which transport cyst antigens to the plasma membrane for release to the nascent cyst wall by exocytosis (21, 22). It is possible that encystation-associated HSP60-HSP70-like proteins of G. lamblia also function as chaperones during assembly of ESV and export of cyst antigens by preventing premature folding or polymerization of proteins which are destined to become part of the microfibrillar cyst wall (8).

We now have identified certain encystation-specific antigens which cross-react with antisera to three different stress proteins. However, the exact relationships between the Giardia antigens and the HSP60 and HSP70 families of stress proteins remain to be elucidated.

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