FOLIA PARASITOLOGICA 45: 117-127, 1998

Characterization and function of the microsporidian polar tube: a review

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Key words: Microsporidia, polar tube proteins, invasion mechanism, *Encephalitozoon*, *Glugea*, protein purification

Abstract. Microsporidia are eukaryotic, obligate intracellular organisms defined by their small spores containing a single polar tube that coils around the interior of the spore. After appropriate stimuli the germination of spores occurs. Conditions that promote germination vary widely among species, presumably reflecting the organism's adaptation to their host and external environment as well as preventing accidental discharge in the environment. It appears that calcium may be a key ion in this process. Regardless of the stimuli required for activation, all microsporidia exhibit the same response to the stimuli, that is, increasing the intrasporal osmotic pressure. This results in an influx of water into the spore accompanied by swelling of the polaroplasts and posterior vacuole. The polar tube then discharges from the anterior pole of the spore in an explosive reaction and is thought to form a hollow tube by a process of eversion. If the polar tube is discharged next to a cell, it can pierce the cell and transfer the sporoplasm into this cell. Polar tubes resist dissociation in detergents and acids but dissociate in dithiothreitol. We have developed a method for the purification of polar tube proteins (PTPs) using differential extraction followed by reverse phase high performance liquid chromatography (HPLC). This method was used to purify for subsequent characterization PTPs from *Glugea americanus*, *Encephalitozoon cuniculi*, *E. hellem* and *E. intestinalis*. These proteins appear to be members of a protein family that demonstrate conserved characteristics in solubility, hydrophobicity, mass, proline content and immunologic epitopes. These characteristics are probably important in the function of this protein in its self assembly during the eversion of the polar tube and in providing elasticity and resiliency for sporoplasm passage.

Microsporidia are protozoa classified in the phylum Microspora that are eukaryotic, obligate intracellular, spore-forming parasites (Levine et al. 1980). They are ubiquitous in the animal kingdom with hundreds of species capable of parasitizing a wide range of invertebrate and vertebrate hosts, including humans (Canning and Lom 1986, Cali and Owen 1988). The spores produced by microsporidia are the identifying characteristic of the phylum. They are small (1-12 μm), unicellular, with a resistant spore wall, a uninucleate or binucleate sporoplasm, and an extrusion apparatus consisting of a single polar tube with an anterior attachment complex (Levine et al. 1980). The polar tube is divided into two regions: the anterior straight portion surrounded by a lamellar polaroplast and attached to the inside of the anterior end of the spore by an anchoring disc; and the posterior coiled region that forms from 4 to approximately 30 coils around the sporoplasm in the spore, depending on the species (Huger 1960, Vávra et al. 1966, Cali and Owen 1988). While inside the spore, the core of the polar tube contains a fine, particulate, electron dense material, and consequently the polar tube is sometimes referred to as a polar filament prior to discharge (Lom and Vávra 1963, Vávra et al. 1966, Lom 1972, Weidner 1972, 1976). When triggered by appropriate stimuli, the polar tube rapidly discharges from the anterior pole of the spore forming a hollow tube that remains attached to the spore (Oshima 1937, 1966, Gibbs 1953, Kramer 1960, Lom and Vávra 1963, Ishihara 1968, Weidner 1972, Frixione et al. 1992).

Polar tubes range from 50-150 μm in length and 0.1-0.2 μm in diameter (Kudo and Daniels 1963, Weidner 1976, Frixione et al. 1992) with coils closest to the anterior end being larger in diameter (Kudo and Daniels 1963, Sinden and Canning 1974). The polar tube has some flexibility in that it shows variation in diameter from 0.10-0.25 μm during discharge (Scar- borough-Bull and Weidner 1985), its diameter can increase to 0.4 μm during sporoplasm passage (Lom and Vávra 1963, Ishihara 1968, Weidner 1972, 1976), and its length shortens by 5-10% after sporoplasm passage (Frixione et al. 1992). The hollow discharged tubes appear to be two to three times as long as the dense, coiled tube inside the spore, and it has been suggested that the internal contents of the tube are incorporated at its growing tip during discharge (Weidner 1972, 1982, Frixione et al. 1992). After complete discharge of the polar tube, the sporoplasm flows through the tube and appears as a droplet at its distal end (Oshima 1937, Gibbs 1953, Kramer 1960, Lom 1972, Weidner 1972,

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Frixione et al. 1992). This process serves as a unique mechanism of infection since the polar tube can pierce an adjacent cell resulting in sporoplasm transfer directly into the host cell cytoplasm (Oshima 1937, Gibbs 1953, Lom and Vávra 1963, Oshima 1966, Ishihara 1968, Weidner 1972, Iwano and Ishihara 1989).

PROPERTIES OF POLAR TUBE PROTEINS (PTPS)

Early studies on the properties of polar tubes found them to be insoluble in water and saliva (Kudo 1921) but completely digested by trypsin in 24 hours (Zwolfer 1926). Polar tubes have been observed to be rapidly digested after extrusion in digestive fluid or in the midgut of insects (Oshima 1927, 1937, Undeen 1976, Undeen and Epsky 1990). Further studies revealed that the polar tube has unusual solubility properties in that it resists dissociation in 1-3% SDS, 1% Triton X-100, 1-10% H_20_2, 5-8 N H_2SO_4, 1-2 N HCl, chloroform, 1% guanidine HCl, 0.1 M proteinase K, 8-10 M urea, 50 mM $NaCO_3$, and 50 mM $MgCl_2$ (Weidner 1972, 1976, 1982). The polar tube, however, dissociates in various concentrations of 2-mercaptoethanol (2-ME), dithiothreitol (DTT), 6% urea with 0.1 M proteinase K and 1% guanidine HCl in 0.1M proteinase K (Weidner 1976, 1982, Keohane et al. 1996a). The polar tube inside the spore has been found to react in the same manner to reducing agents and detergents as everted polar tubes (Weidner 1976, 1982).

It is the unusual solubility properties of polar tube proteins that allow their separation from other proteins in the spore. Since spore coat and polar tube are resistant to concentrations of detergents and acids that solubilize most other proteins, treatment of spores with these agents can be used to isolate PTPs. In one such protocol (Weidner 1976), polar tubes were released from the resistant spores either by triggering spore discharge and release of sporoplasm or by disrupting the spore wall with 8 N sulfuric acid. The spores were then washed with 3% SDS to remove sporoplasm and soluble spore coat proteins. Subsequent treatment with 1% DTT or 50% 2-ME solubilized the polar tubes but left the spore coats intact. When this procedure was performed on one species of microsporidia, *Ameson michaelis,* SDS-polyacrylamide gel electrophoresis (PAGE) of the DTT or 2-ME supernatants of the spore preparation demonstrated a single 23-kDa protein band, presumably representing a polar tube protein (Weidner 1976). Amino acid analysis of this protein demonstrated the presence of multiple cysteine residues (Weidner 1976). Since both DTT and 2-ME readily dissociate the polar tube, disulfide bridging may be important in stabilizing PTPs and polar tube structure.

A 43 kDa PTP was isolated from the spores of the fish microsporidium, *Glugea americanus* utilizing a similar extraction procedure (Keohane et al. 1994). After sequentially extracting glass bead disrupted spores with 1% SDS and 9 M urea, followed by solubilization of the residual polar tubes in 2% DTT (Keohane et al. 1996a) (see Figs. 1-3), the DTT solubilized material contained four protein bands of 23, 27, 34 and 43 kDa. The PTPs in the DTT supernatant had no immunoblot reactivity with commercial anti-actin, anti-β tubulin or anti α-tubulin selected for their reactivity across phyla. A monoclonal antibody (mAb) 3C8.23.1, reactive to the 43 kDa by immunoblotting, strongly localized to the polar tubes of *G. americanus* spores by immunogold electron microscopy indicating it was polar tube in origin (see Fig. 4). Subsequently, the 43 kDa PTP in the DTT solubilized material was purified to homogeneity using reverse phase high performance liquid chromatography (HPLC) (Keohane et al. 1996b) (see Fig. 5). This purified fraction migrated at 43 kDa by SDS-PAGE and silver staining and reacted with polar tube mAb 3C8.23.1 by immunoblotting (Keohane et al. 1996b) (see Fig. 5 insert: lanes A and B). Polyclonal sera from mice immunized with the purified 43 kDa PTP reacted to a 43 kDa protein in *G. americanus* spore lysate by immunoblotting (see Fig. 5 insert: lane C), and intrasporal and extruded polar tubes of *G. americanus* by immunogold electron microscopy. Amino acid analysis revealed this PTP to be proline rich.

Applying this PTP purification protocol to the microsporidia associated with human infections (Keohane et al. 1996c), the polar tubes of *Encephalitozoon intestinalis*, *E. hellem* and *E. cuniculi* were found to have the same solubility properties as those of *G. americanus*, that is they were insoluble in 1% SDS and 9 M urea and soluble in 2% DTT. The DTT-solubilized material from all three microsporidia demonstrated a UV-absorbing peak at a similar retention time as *G. americanus* PTP, indicating similar hydrophobicity. On SDS-PAGE and silver staining, the HPLC purified PTP of *E. hellem* migrated at 55 kDa, while *E. cuniculi* and *E. intestinalis* migrated at 45 kDa. Polyclonal serum from a rabbit immunized with the purified PTP of *E. hellem* (anti-PTP Eh55) reacted by immunoblotting with the purified PTPs of *E. hellem*, *E. cuniculi*, *E. intestinalis* and *G. americanus*. Anti-PTP Eh55 also strongly localized to intrasporal and extruded polar tubes of *E. hellem* by immunogold electron microscopy. Amino acid analysis of the PTPs of the human microsporidia also revealed a high proline content (Keohane et al. 1996c). The *E. hellem* 55 kDa PTP has recently been cloned and it displays the proline content predicted from the amino acid analysis of the protein (Weiss and Keohane – unpubl. data). It appears that these PTPs are members of a protein family that have conserved solubility, hydrophobicity, mass, proline content and immunologic epitope characteristics.

Proline is a hydrophobic imino acid. When it is present in a polypeptide, it forms a fixed kink in the structure resulting in rigidity of the chain. High proline content is a feature of several structural proteins

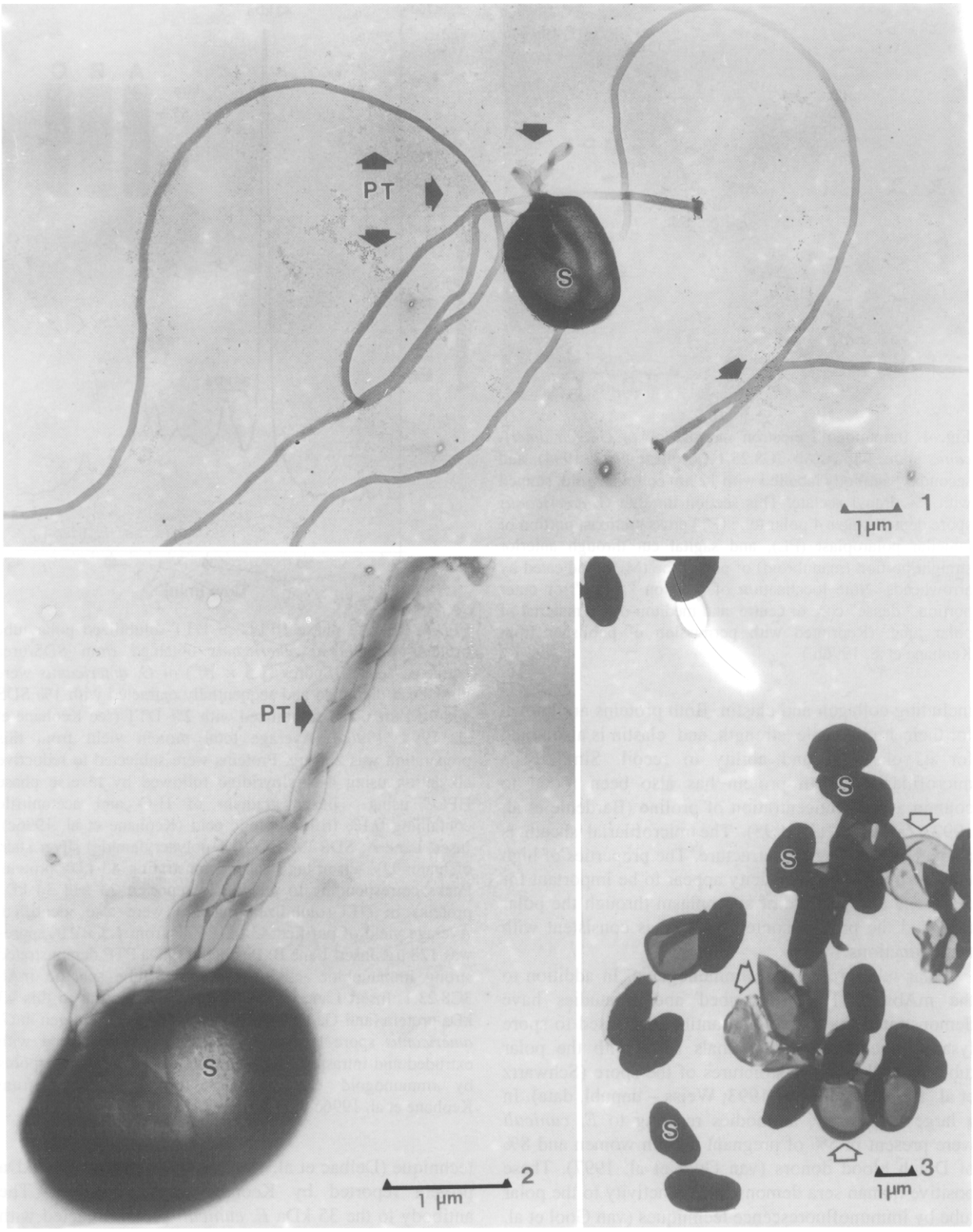

Fig. 1-3. Negative stain transmission electron microscopy using Formvar and carbon coated grids stained with 0.5% uranyl acetate or 1% phosphotungstic acid of spores of *Glugea americanus* disrupted with 0.5 mm acid washed glass beads (Sigma, St. Louis, MO) for 4 min on a Mini Beadbeater (Biospec Products, Bartlesville, OK). **Fig. 1**, **2.** Disrupted spores after extracting five times with 1% SDS and once with 9 M urea. Note broken spores (S) and straight and twisted polar tubes (PT, closed arrows). **Fig. 3.** Disrupted spores after washing five times with 1% SDS, once with 9 M urea, and incubated 2 h with 2% DTT. Note broken spores (S), lack of spore contents (open arrows), and absence of polar tubes. (Reprinted with permission of the publisher from Keohane et al. 1996b.)

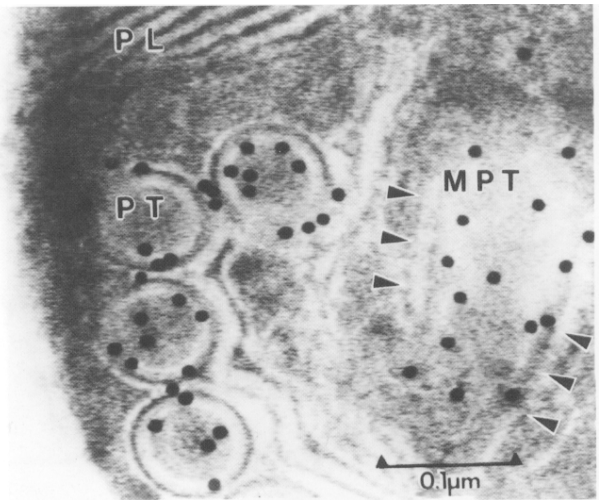

Fig. 4. Immunogold electron microscopy of *Glugea americanus* spore with mAb 3C8.23.1 (Keohane et al. 1994), and secondary antibody labelled with 12 nm colloidal gold, stained with 1% uranyl acetate. This section through *G. americanus* spore demonstrates 4 polar tube (PT) cross sections, portion of lamellar polaroplast (PL), and sagital cut through anterior straight portion (manubroid) of polar tube (MPT) indicated by arrowheads. Note localization of gold on "sheath" or outer portion, "dense" core or centre, and medium-dense material of polar tube. (Reprinted with permission of publisher from Keohane et al. 1996b.)

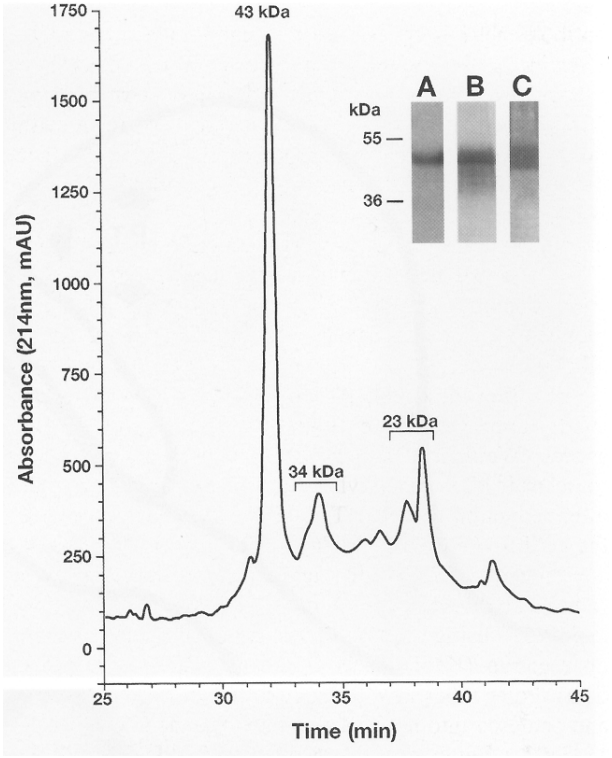

Fig. 5. Reverse phase HPLC of DTT-solubilized polar tube proteins of *Glugea americanus* obtained from SDS/urea extracted spores. Spores (1.5×10^8) of *G. americanus* were glass bead disrupted and sequentially extracted with 1% SDS and 9 M urea, and solubilized with 2% DTT (see Keohane et al. 1994, 1996a). Average total protein yield from this preparation was 200 µg. Proteins were subjected to reductive alkylation using 4-vinylpyridine followed by reverse phase HPLC using linear gradient of H_2O and acetonitrile containing 0.1% trifluoroacetic acid (Keohane et al. 1996c). Insert Lane A: SDS-PAGE (10% polyacrylamide) silver stain of major UV absorbing peak demonstrating 43 kDa protein. Peaks corresponding to previously reported 23 and 34 kDa proteins in DTT-solubilized material were also identified. Average yield of purified 43 kDa PTP from 1.5×10^8 spores was 128 µg. Insert Lane B: Purified 43 kDa PTP demonstrated strong immunoblot activity with polar tube specific mAb 3C8.23.1. Insert Lane C: Polyclonal mouse serum to this 43 kDa protein (anti-Ga PTP_{43}) reacted with 43 kDa antigen in *G. americanus* spore lysate. Anti-Ga PTP_{43} also reacted with extruded and intrasporal polar tubes of *G. americanus* spores by immunogold electron microscopy. (Reprinted from Keohane et al. 1996c with permission of Elsevier).

including collagen and elastin. Both proteins are known for their high tensile strength, and elastin is also noted for its elasticity and ability to recoil. Similarly a microfiliarial sheath protein has also been found to contain a high concentration of proline (Bardehle et al. 1992, Zahner et al. 1995). The microfilarial sheath is also a flexible, bag-like structure. The properties of high tensile strength and elasticity appear to be important for discharge and passage of sporoplasm through the polar tube and the proline content of PTP is consistent with these functions.

Polar tube proteins are immunogenic. In addition to the mAbs to PTPs described above, studies have demonstrated that polyclonal antibodies raised to spore lysates in experimental animals react with the polar tube, as well as other structures of the spore (Schwartz et al. 1993, Zierdt et al. 1993; Weiss – unpubl. data). In a large serosurvey, antibodies reacting to *E. cuniculi* were present in 5% of pregnant French women and 8% of Dutch blood donors (van Gool et al. 1997). These positive human sera demonstrated reactivity to the polar tube by immunofluorescence techniques (van Gool et al. 1997). In one study of the immunologic response to spore antigens of *Glugea atherinae* and *E. cuniculi* several candidate PTPs were identified (Delbac et al. 1996). Proteins of 34, 75, and 170 kDa in *G. atherinae* and 35, 52 and 150 kDa in *E. cuniculi* were localized to the polar tube by immunogold electron microscopy. It is possible that the 52kDa protein identified by this technique (Delbac et al. 1996) is the same as the 45kDa protein reported by Keohane et al. (1996c). The antibody to the 35 kDa *E. cuniculi* protein reacted with *G. atherinae* polar tube by immunogold electron microscopy confirming that shared PTP epitopes must exist (Delbac et al. 1996). Cross-reaction to the polar tube of *Spraguea lophii* was also demonstrated by these immunofluorescence techniques (Delbac et al. 1996). In the process of eversion of the polar tube unique immunologic epitopes may be exposed. Monoclonal

antibody Si91 is specific for extruded polar tubes of *E. intestinalis* by immunogold electron microscopy and recognizes 60 and 120 kDa bands on western blotting (Beckers et al. 1996). This mAb does not react with polar tubes (i.e. filaments) within the spore. Further evidence for the high specificity of some PTP epitopes is the absence of cross-reactivity of mAb Si91 for extruded polar tubes of *E. hellem* (Beckers et al. 1996).

A study of the assembly properties of an isolated PTP, demonstrated that SDS washed tubes of PTP, reduced by 2-ME, unalkylated, and dialyzed against an alkaline buffer, remained dissociated, upon acidification, however, PTP reassembled into sheets or shells, appearing more fluid than PTP of discharged spores (Weidner 1976). Reassembly was not observed to occur if PTP was alkylated after 2-ME treatment, nor after reduction by 1% DTT and subsequent removal of the DTT (Weidner 1976). We found that DTT solubilized PTP would aggregate when DTT was removed by dialysis, but reductive alkylation of cysteine residues using 4-vinylpyridine prevented such aggregation (Keohane et al. 1996a; Weiss – unpubl. data). Polar tubes have been reported to show branches and coalesce into networks when suspended in 0.05-0.1 M $CaCl_2$ (Weidner 1982). A putative 52 kDa PTP of *E. cuniculi* has been demonstrated to bind $^{45}Ca^{2+}$ (Delbac et al. 1996). These properties suggest that both disulfide bonds and calcium may play a role in the assembly or function of PTPs.

POLAR TUBE GERMINATION (ACTIVATION)

Polar tube extrusion was first reported by Thelohan (1892, 1894) and subsequently confirmed by others (Stempel 1909, Korke 1916, Kudo 1918). The polar tube discharges from the anterior pole of the spore in an explosive reaction occurring in less than 2 seconds (Oshima 1937, Lom and Vávra 1963, Vávra et al. 1966, Weidner 1972, Frixione et al. 1992) and is thought to form a hollow tube by a process of eversion, similar to everting the finger of a glove (Oshima 1937, 1966, Gibbs 1953, Lom and Vávra 1963, Ishihara 1968, Lom 1972, Weidner 1982). Spore discharge is generally believed to occur in several phases: 1) activation, 2) increase in intrasporal osmotic pressure, 3) eversion of the polar tube, and 4) passage of sporoplasm through the polar tube. The exact mechanism of the process of spore discharge is not understood.

Conditions that activate spores vary widely among species, presumably reflecting the organisms' adaptation to their host and external environment (Undeen and Epsky 1990). Since microsporidia are found in a wide range of terrestrial and aquatic hosts, different species may require unique activation conditions for spore discharge. These specific conditions are probably important to prevent accidental discharge in the environment (Undeen and Avery 1988a, Undeen and Epsky 1990) and may contribute to host specificity.

Conditions that have been shown to promote spore discharge include incubation at an alkaline pH (Ishihara 1967, Undeen 1978, Undeen and Avery 1984, Undeen and Epsky 1990), acidic pH (Korke 1916, Hashimoto et al. 1976, Undeen 1978, 1983), or a pH shift from acid to alkaline (Pleshinger and Weidner 1985) or from alkaline to less alkaline or neutral (Oshima 1964, Undeen 1978, Malone 1984). Other species have demonstrated spore discharge at both acidic and alkaline conditions (Hashimoto et al. 1976, Undeen 1983, Undeen and Avery 1988a). Dehydration by drying or hyperosmotic solutions followed by rehydration has been effective in promoting spore discharge in some species (Gibbs 1953, Kramer 1960, Olsen et al. 1986, Whitlock and Johnson 1990), while dehydration followed by rehydration at an alkaline pH was effective in others (Weidner 1972, Undeen 1978, Undeen and Epsky 1990). Various cations including potassium, lithium, sodium, cesium (Ishihara 1967, Undeen 1978, Malone 1984, 1990, Undeen and Epsky 1990, Whitlock and Johnson 1990, De Graaf et al. 1993, Frixione et al. 1994) and anions such as bromide, chloride, iodide and fluoride (Undeen and Avery 1988a) have been used to promote discharge. It is apparent from these studies that both cations and anions enter the spore passively. Mucin or polyanions (Pleshinger and Weidner 1985, Weidner et al. 1995), hydrogen peroxide (Kudo 1918, Lom and Vávra 1963, Hashimoto et al. 1976, Leitch et al. 1993), low dose ultraviolet radiation (Undeen and Vandermeer 1990), a Na^+ ionophore monensin (Frixione et al. 1994) and calcium ionophore A 23187 (Weidner and Byrd 1982) have also been used to trigger discharge. While the spore wall forms a barrier to larger molecules (i.e. it functions as a molecular sieve), it appears that alkali metal cations freely pass through the spore wall and plasma membrane of the spore. These cations are required for the germination process and that, to some extent, the smaller cations are more effective (Frixione et al. 1994).

Inhibitors of spore discharge include: concentrated alcohols (Kudo 1918), 0.01-0.1 M magnesium chloride (Malone 1984), ammonium chloride (Undeen 1978, Undeen and Avery 1988b, Undeen and Epsky 1990), low salt concentrations (10-50 mM) (Undeen 1978), sodium fluoride (Undeen and Avery 1988a), silver ions (Ishihara 1967), gamma radiation (Undeen et al. 1984), ultraviolet light (Whitlock and Johnson 1990), temperatures greater than 40°C (Whitlock and Johnson 1990), calcium channel antagonists (lanthanum, verapamil, nefedipine), calmodulin inhibitors (chlorpromazine, trifluroperazine) (Pleshinger and Weidner 1985, Leitch et al. 1993), a microfilament disrupter (cytochalasin D), a microtubule disrupter (demecolcine) and itraconazole (Leitch et al. 1993). Calcium chloride (0.001 to 0.1M) has been found to

inhibit spore discharge in some studies (Oshima 1964, Ishihara 1967, Undeen 1978, Weidner and Byrd 1982, Malone 1984), while 0.2 M $CaCl_2$ at pH 9.0 (Pleshinger and Weidner 1985) and 1 mM $CaCl_2$ (Leitch et al. 1993) promoted discharge in other studies. EGTA in the presence of calcium also promoted spore discharge in one study (Malone 1984) and inhibited discharge in another (Pleshinger and Weidner 1985). Removal of clathrin and calmodulin from the intermediate filament cage assembly which envelopes the spores of *Spraguea lophii*, resulted in irreversible inactivation of spore discharge (Weidner 1992). These studies suggest that calcium and the pH of the spore play an important part in the germination process.

It has been theorized that, regardless of the mode of activation, microsporidia exhibit the same response to the stimuli, that is, increasing the intrasporal osmotic pressure (Kudo 1918, Oshima 1937, Lom and Vávra 1963, Undeen 1990, Undeen and Frixione 1990, Frixione et al. 1992). This increase in osmotic pressure results in an influx of water into the spore accompanied by swelling of the polaroplasts and posterior vacuole prior to spore discharge (Huger 1960, Lom and Vávra 1963, Weidner and Byrd 1982, Frixione et al. 1992). It is this pressure which forces the eversion of the polar tube and expulsion of the sporoplasm (Undeen 1990). The spore wall provides structural resistance, as well as elasticity and flexibility for this process (Undeen and Frixione 1990, 1991). In hyperosmotic solutions, polar tube discharge is inhibited or slowed down (Oshima 1937, Lom and Vávra 1963, Undeen and Frixione 1990, Frixione et al. 1992), and sporoplasm passage does not occur (Weidner 1976, Frixione et al. 1992), thus providing indirect evidence for the osmotic pressure theory. Water flow across the spore wall and plasma membrane is a clear requirement for osmotic theories of spore discharge. A recent study using D_2O has demonstrated that water influx into spores occurs through a specific transmembrane pathway (i.e., an aquaporin) sensitive to $HgCl_2$ and the hydration state of the membrane (Frixione et al. 1997). D_2O inhibits spore dicharge in a concentration dependent manner (Frixione et al. 1997). The inhibition of germination by D_2O could be overcome by either an increase in temperature or ionic strength both of which are associated with a decrease in water structure and thus an opening of such aquaporins (Frixione et al. 1997). Supporting evidence for the presence of an aquaporin in the plasma membranes of spores is the presence of 7-10 nm intramembrane particles of uniform appearance similar to monomeric aquaporins (Liu and Davis 1973, Vávra et al. 1986, Undeen and Frixione 1991).

Several theories have been proposed as to the mechanism for increasing the osmotic pressure in the spore. One of the earliest explanations was that the activation simply increases the permeability of the spore coat to water (Lom and Vávra 1963). However, data suggest while the spore coat functions as a molecular sieve, it is freely permeable to water. Another theory involved the creation of a proton gradient by the alkaline environment surrounding the spore (Dall 1983). The proton gradient drives a proton-cation exchange mechanism consisting of a carboxylic acid ionophore. As protons in the sporoplasm are depleted, the increase in alkalinity triggers the same mechanisms in the membrane of organelles, particularly the polaroplast and posterior vacuole. Water flows into the spore, due to the generalized osmotic imbalance, increasing the intrasporal pressure (Dall 1983). It should be noted, however, that not all microsporidia require an alkaline pH for spore discharge and the cationic flux described does not appear sufficient for the osmotic pressure required for germination. Microsporidian spores have been reported to contain trehalose and trehalase (Wood et al. 1970, Vandermeer and Gochnauer 1971). An alternative theory for spore discharge is based on the finding of decreased trehalose levels in discharged spores as compared to undischarged spores of *Nosema algerae* (Undeen et al. 1987, Undeen and van der Meer 1994). In this mechanism, activation causes changes in the spore which brings the trehalose in contact with the enzyme trehalase, perhaps by a disruption of compartments within the spore (Undeen 1990). The trehalose is degraded into a larger number of small molecules, causing an increase in osmotic pressure. The subsequent flow of water into the spore results in increase in intrasporal pressure and spore discharge (Undeen 1990). However, in *Nosema apis* DeGraaf et al. (1993) found little difference in the trehalose/glucose ratio before and after germination suggesting that this mechanism may not apply to all microsporidia. Calcium has also been proposed to play a major role in spore discharge, in which its displacement from the polaroplast membrane would either activate a contractile mechanism or combine with the polaroplast matrix causing polaroplast swelling (Weidner and Byrd 1982). Calcium ionophore A23187, sodium citrate and phosphate were found to trigger polaroplast swelling and polar tube discharge, while calcium chloride inhibited the reaction (Weidner 1982, Weidner and Byrd 1982).

Spore discharge is a complex process requiring several steps before the polar tube begins to evert and transfer of sporoplasm to its host cell can occur. One possible synthesis of the current data is that ion flux into the spore occurs in response to external stimuli (such as pH change) and that this ionic flux results in the displacement of calcium from intramembranous compartments. The displacement of calcium leads to an activation of an enzyme (with a specific pH requirement), such as trehalase, which amplifies the hydrostatic force driving water into the spore by the breakdown of a complex molecule (trehalose) to its components (glucose and its subsequent metabolites)

resulting in an increase in the number of osmotically active molecules. The influx of water causes swelling of the posterior vacuole and polaroplast and eventually rupture of the spore through the anterior attachment complex with eversion of the polar tube. Inhibition of spore germination could thus occur by interfering with this process at any one of these steps. For example, an alteration in spore pH by ammonium chloride could inhibit ion flux and/or enzyme activity. Stimulation by low dose ultraviolet radiation or hydrogen peroxide may be due to damage of intracellular compartments allowing enzyme and substrate to interact bypassing the calcium activation mechanism.

POLAR TUBE DISCHARGE

The first sign of spore discharge is a visible protrusion at the anterior end of the spore at the polar cap (Lom and Vávra 1963, Kudo and Daniels 1963, Frixione et al. 1992), which is followed by the rapid emergence of the polar tube in a helicoidal fashion along nearly a straight line (Frixione et al. 1992). Full discharge of the polar tube requires less than 2 seconds, with a maximum velocity of 105 µm/s (Frixione et al. 1992). Polar tubes range in size from 50-150 µm in length and 0.1-0.2 µm in diameter (Kudo and Daniels 1963, Weidner 1976, Frixione et al. 1992). The same polar tube thickness has been observed before and after discharge, including discharge into media of various viscosities (Weidner 1976). Inside the spore the polar tube is filled with electron dense material (believed to be unassembled polar tube protein), while the discharged tubes appear as hollow cylinders (Weidner 1976, 1982). The intrasporal polar tube is surrounded by a membrane separating it from the sporoplasm (Weidner 1972, 1976). Sinden and Canning (1974) suggested that this was a double membrane with the outer membrane being contiguous with the polar sac (i.e. the anchoring disk). Discharged polar tubes have a sheath which is sensitive to trypsin, is silver methenamine negative and is able to bind ferritin-conjugated concanavalin A (Weidner 1972). Incompletely discharged tubes appear as a cylinder within a cylinder (i.e. double membrane cylinders) at their distal ends (Weidner 1982, Weidner et al. 1994, 1995). A homogenous pattern of subunits has been observed in completely and incompletely discharged tubes, and appears identical to the material inside the tube (Weidner 1976, 1982). Evidence suggests that PTPs within the polar filament are on the outside of the polar tube after discharge as well as that polaroplast and polar filament membranes are inside the hollow tube (Weidner 1982, Weidner et al. 1984, 1995). After eversion, the polar tube remains attached to the anterior end of the spore (Ishihara 1968). Discharged tubes appear to be about two to three times as long as the coiled tube inside the spore (Lom and Corliss 1967, Weidner 1972), and it has been suggested that the internal contents of the tube are incorporated at its growing tip as it is being everted (Oshima 1966, Weidner 1982, Frixione et al. 1992). This theory is supported by observations using pulse labelling with latex particles (Weidner 1982) and by video interference contrast microscopy (Frixione et al. 1992, Weidner et al. 1994, 1995). It has also been observed that the portion of the tube already everted remains unchanged while the tube elongates and even changes direction at the tip (Weidner 1982, Frixione et al. 1992). It should be noted that spores can be broken by mechanical pressure (Kudo 1918, Kudo and Daniels 1963, Weidner 1982, Dall 1983, Undeen 1990) or by glass bead disruption (Connor 1970, Langley et al. 1987, Keohane et al. 1996a), thus releasing polar tubes from the sides of the spores. Polar tubes display similar ultrastructural appearances, i.e. they form a filament, regardless of whether they were triggered to activate and evert from the apical pole, or if they passively burst through the lateral walls of the spores (Weidner 1982).

After complete discharge of the polar tube, the sporoplasm flows through the polar tube and appears as a droplet at its distal end (Oshima 1937, Gibbs 1953, Lom 1972, Weidner 1972, Frixione et al. 1992). During this passage through the polar tube sporoplasm and nucleus leave their plasma membrane behind in the spore (Weidner 1976, Undeen and Frixione 1991) and acquire a new limiting membrane from the polaroplast (Weidner et al. 1984, 1995). Using video enhanced contrast microscopy, there is a time delay between completion of discharge and appearance of the droplet of about 15-500 ms (Frixione et al. 1992). It has been suggested that the delay might be due to the eversion of a blind ended tube which needs to be opened by some mechanism prior to sporoplasm release (West 1960, Erickson et al. 1968, Frixione et al. 1992). Sporoplasm passage has not been observed in partially discharged tubes (Gibbs 1953, Weidner 1972, Frixione et al. 1992). While in contact with the tip of the polar tube, the sporoplasm droplet enlarges to a volume in excess of what might be expected from the size of the spore (Frixione et al. 1992). This is most likely due to the movement of water into the sporoplasm secondary to an osmotic gradient between the sporoplasm and the environment (Frixione et al. 1992). The polar tube has some flexibility in that it shows variation in diameter from 0.1-0.25 µm during eversion (Scarborough-Bull and Weidner 1985), its diameter can increase to 0.4 µm during sporoplasm passage (Lom and Vávra 1963, Oshima 1966, Ishihara 1968, Weidner 1972, 1976), and its length shortens by 5-10% after sporoplasm passage (Frixione et al. 1992).

If the polar tube is discharged next to a cell, it can pierce the cell and transfer, i.e. inject, the sporoplasm into that cell (Oshima 1937, Gibbs 1953, Lom and Vávra 1963, Oshima 1966, Ishihara 1968, Weidner 1972, Iwano and Ishihara 1989). If there are no adjacent cells, the droplet of sporoplasm remains attached to the

polar tube for a period of time. The plasma membrane of the spore is left attached to the inner face of the spore wall during germination (Weidner 1976, Undeen and Frixione 1991). It has been suggested that a new membrane for the discharged sporoplasm is provided by the polaroplast (Weidner et al. 1984). Further, this membrane is essential for the formation and lengthening of the polar tube during extrusion as the polar tube material inside the filament polymerizes on the outside of the membrane (Weidner et al. 1994, 1995). Other authors have indicated that the polaroplast does not participate in polar tube formation (Sinden and Canning 1974, Toguebaye and Marchand 1987). Immunogold electron microscopy also suggests that the polar tube and polaroplast are antigenically distinct (Keohane et al. 1994, 1996a,b,c, Beckers et al. 1996, Delbac et al. 1996). The mechanism by which the polar tube penetrates host cells is unknown. It is also not known if the polar tube binds to a specific receptor on the host cell.

CONCLUSIONS

While descriptions of the polar tube as a unique microsporidian structure occurred over 100 years ago (Thelohan 1892, 1894), the biochemical components of this structure and the mechanism of germination of spores remain to be definitively determined. Some of these proteins appear to be conserved among the microsporidia. The application of the techniques of modern biology (both immunologic and molecular) has resulted in the identification of several candidate polar tube proteins. The interactions and functional significance of these identified proteins remain to be determined. Experimental evidence suggests that osmotic pressure generated in the spore prior to polar tube eversion is a key event in germination. The mechanisms by which this osmotic gradient is generated and the significance of both alkali cations and anions as well as calcium in the initiation of this process remain to be resolved. Work on *Nosema algerae* has established that trehalose may be a key compound in germination (Undeen et al. 1990). Studies of this nature need to be extended to microsporidia found in non-insect hosts to determine whether these findings can be generalized to other microsporidia. The polar tube serves as a unique vehicle for transmission of infection by piercing an adjacent host cell, thereby inoculating the sporoplasm directly into that cell, functioning essentially as a hypodermic needle. Additional characterization of the early events in the rupture of the anterior attachment complex as well as the eversion of the polar tube are needed. The invasion organelle of the microsporidia (polar tube and polaroplast) has successfully served this diverse phylum, resulting in a group of obligate intracellular organisms capable of infecting almost any cell type. Further study may lead to novel strategies for control of these important parasitic protozoa.

Acknowledgments. Supported by NIH AI31788 and UMDNJ Foundation Grant 20-97. With thanks to Murray Wittner, M.D., Ph.D. and Herbert B. Tanowitz, M.D. for advice on this manuscript. George A. Orr, Ph.D. for advice and assistance on protein purification methods. Ann Cali, Ph.D. and Peter M. Takvorian, Ph.D. for advice and assistance with immunogold electron microscopy.

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Received 16 July 1997

Accepted 30 October 1997