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Functional characterization of a putative aquaporin from *Encephalitozoon cuniculi*, a microsporidia pathogenic to humans

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Abstract

The microsporidia are a group of obligate intracellular parasitic protists that have been implicated as both human and veterinary pathogens. The infectious process of these organisms is believed to be dependent upon the rapid influx of water into spores, presumably via aquaporins (AQPs), transmembrane channels that facilitate osmosis. An AQP-like sequence of the microsporidium *Encephalitozoon cuniculi* (EcAQP), when cloned and expressed in oocytes of Xenopus laevis, rendered these oocytes highly permeable to water. No permeability to the solutes glycerol or urea was observed. Pre-treatment of EcAQP-expressing oocytes with $HgCl_2$ failed to inhibit their osmotic permeability, as predicted from EcAQP's lack of mercury-sensitive cysteine residues near the NPA motifs which line the AQP aqueous pore. EcAQP exhibits sequence identity to AQP A of *Dictyostelium discoideum* (26%) and human AQP 2 (24%). Further study of AQPs in microsporidia and their potential inhibitors may yield novel therapeutic agents for microsporidian infections.

Keywords

Microsporidia; Aquaporin; Germination; Water permeability; Encephalitozoon; Xenopus oocytes

1. Introduction

The phylum Microsporidia, a group of obligate intracellular parasitic protists, contains over 1200 species representing nearly 150 genera (Weiss, 2001; Garcia, 2002). Since the mid-1980s, these organisms have been increasingly implicated as agents of human disease,

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especially in their capacity as opportunistic parasites of AIDS patients (Cali, 1991; Weiss, 2001) and other immunosuppressed individuals (Franzen and Muller, 2001). The most common clinical manifestation is chronic diarrhea and wasting due to enteric infection, but the spectrum of disease is wide and includes most organ systems (Cali, 1991; Franzen and Muller, 2001). Albendazole and other benzimidazoles that bind tubulin, as well as fumagillin and its derivatives that inhibit methionine aminopeptidase type 2, have been identified as being useful agents for the treatment of microsporidiosis; however, effective and safe treatments do not yet exist for some of the microsporidian species that infect humans (Costa and Weiss, 2000).

Microsporidia produce an infectious, environmentally resistant spore, which is capable of extruding its internal polar filament and thereby inoculating its contents into a nearby host cell. The mechanism of spore germination has long been of interest to microsporidian researchers, and naturally it has been suggested that inhibition of this process would constitute an effective anti-microsporidian therapy (Keohane and Weiss, 1999). Germination stimuli are variable (Undeen and Epsky, 1990) but are postulated (reviewed in Keohane and Weiss, 1999) to result in cleavage of the disaccharide trehalose into glucose and its metabolites, producing an increase in intrasporal pressure and osmotic swelling that leads to extrusion of the polar filament. Because water flux across the lipid bilayer is limited, it has been suggested that microsporidia may possess aquaporins (AQPs) (Frixione et al., 1997), integral membrane channel proteins that facilitate osmosis (Verkman and Mitra, 2000; Agre and Kozono, 2003). This suspicion was bolstered by the observation that germination of *Brachiola (Nosema) algerae* spores is inhibitable by mercury salts (Frixione et al., 1997), which inhibit AQP function (Yang, 2000; Agre and Kozono, 2003). Further evidence suggesting the presence of AQPs in microsporidia surfaced with the identification of a single AQP-like sequence within the genome of *Encephalitozoon cuniculi*, a human-pathogenic species (Katinka et al., 2001).

The present study was undertaken to functionally characterise the putative *E. cuniculi* AQP (*Ec*AQP) protein. Aquaporin function is typically assayed in RNA-injected *Xenopus* oocytes, where swelling occurs under osmotic stress due to expression of the exogenous AQP (Verkman and Mitra, 2000; Agre and Kozono, 2003). Utilizing this assay, the osmotic permeability and solute conductivity of *Ec*AQP-injected oocytes were investigated. In addition, the *Ec*AQP amino acid sequence was compared to those of human, plant, fungal, and other protist parasite AQPs.

2. Materials and methods

2.1. Parasites

Encephalitozoon cuniculi was cultured in RK13 cells (rabbit kidney cells CCL37; American Type Culture Collection, Rockville, Md.) at 37 8C and 5% CO_2. Infected RK13 cells were maintained in continuous culture in minimum essential medium supplemented with 7% heat-inactivated FCS, 1% penicillin–streptomycin and 1% amphotericin B (Fungizone; Invitrogen, Carlsbad, CA) and subpassaged every week by trypsin-EDTA treatment (Invitrogen, Carlsbad, CA). Spores were harvested from culture medium twice weekly.

2.2. Cloning and expression of EcAQP

Genomic DNA was isolated from disrupted spores of *E. cuniculi* by SDS and proteinase K treatment and homogenization, followed by phenol–chloroform extraction, as previously described (Keohane et al., 1998). Recognition sites for the restriction enzymes *Xma* I and *Xba* I were engineered onto the N- and C-termini, respectively, of *Ec*AQP (GenBank accession no. NP_586002), by PCR-amplification of genomic DNA. PCR was performed

using *Pfx* DNA polymerase and 15 μM of each primer (primers, restriction sites are italicised: 5′GGACCT*CCCGGG*ATGACCAGAGAGACATTGAAG3′ (forward), 5′GACCC*TCTAGA*CTAAAAGCTGAGCTTGT ACAG3′ (reverse)); DNA was amplified for 35 cycles (45 s denaturation at 94 °C, 45 s annealing at 40 °C and 60 s extension at 60 °C). The amplicon was cloned into the *Xma* I–*Xba* I multiple cloning site of the pGEMHE *Xenopus* expression vector (Liman et al., 1992) by *Xma* I–*Xba* I digestion and mutual ligation of the amplicon and vector, yielding pGEMHE-*Ec*AQP. *Escherichia coli* strain DH5α was subsequently transformed by pGEMHE-*Ec*AQP; large-scale plasmid purification from ampicillin-screened colonies was accomplished by the HiSpeed Kit (Qiagen, Valencia, CA). Identity of pGEMHE-*Ec*AQP was confirmed by restriction digestion analysis and by sequencing. pGEMHE-*Ec*AQP was linearised downstream of the 3′ untranslated region (UTR) by digestion with the restriction enzymes *Sph* I or *Nhe* I. cRNA was generated in vitro by the mMessage mMachine kit (Ambion, Austin, TX) as per manufacturer's instructions using T7 RNA polymerase, nucleotide phosphate (NTP), 7-methyl-guanosine cap analog, and RNase-inhibitor. *Xenopus laevis* maintenance and surgical oocyte removal were performed as previously described (Mak and Foskett, 1994). Defolliculated stages V and VI oocytes were injected with 55 ng in 37 nL of pGEMHE-*Ec*AQP mRNA or 37 nL water (controls). Oocytes were incubated in isoosmotic ND96 buffer/pyruvate (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvate, pH 7.6) at 16 °C for 4–6 days, adding fresh buffer after 3 days.

2.3. Oocyte swelling assays

Swelling assays were conducted at room temperature in plastic 96-well culture plates. For measurement of water permeability, oocytes were transferred to hypotonic source of ND96/pyruvate (1:3 diluted ND96 buffer/0.83 mM pyruvate) or, if indicated, pre-incubated for 5 min in isoosmotic ND96/pyruvate +0.1, 1, 10, 200 μM, or 1 mM $HgCl_2$ and then transferred to hypotonic ND96/pyruvate+$HgCl_2$. For measurement of solute conductivity, oocytes were transferred to ND96/pyruvate where 65 mM NaCl had been replaced with 130 mM glycerol or urea. Swelling was video-monitored every 3 s with a Zeiss SV11 dissecting microscope (Zeiss, Göttingen, Germany) at 66×magnification, and a Retiga 1300 digital camera and IPLab software (Scanalytics, Fairfax, VA). Image analysis was accomplished with ImageJ software (National Institutes of Health, US; URL: http://rsb.info.nih.gov/ij/) by converting images to binary and treating the oocyte as a growing sphere whose volume could be inferred from its cross-sectional area. Water permeability (P_f, cm/s) was calculated based on the first 60 s of the assay and according to the following equation: $P_f=\{[V_0][d(V/V_0/dt]\}/[(S)(V_w)(osm_{in}-osm_{out})]$; V_0 and S are the initial volume and surface area of each individual oocyte, respectively; $d(V/V_0)/dt$, the relative volume increase per unit time; V_w, the molecular volume of water (18 cm^3/mol); and osm_{in-out}, the osmotic gradient between the inside and outside of the oocyte (140×10^{-6} mol/cm^3). Solute conductivity was inferred from $d(V/V_0)/dt$. Water permeabilities and swelling rates were statistically compared with the Student's *t*-test (two-tailed).

2.4. Sequence analysis

The BLAST program (Altschul et al., 1997) was used to search the databases for proteins similar to *Ec*AQP. The Biology Workbench (URL: http://workbench.sdsc.edu/) was employed for Kyle–Doolittle hydropathy analysis (Kyle and Doolittle, 1982; Pearson and Lipman, 1988; Pearson, 1990), transmembrane segment prediction (Persson and Argos, 1994), CTREE phylogenetic construction (algorithm and program: The Biology Workbench, David J. States), and multiple protein sequence alignment (Felsenstein, 1989; Higgins et al., 1992; Thompson et al., 1994).

3. Results

3.1. Functional characterization of EcAQP

Mean swelling rates of *Ec*AQP- and water-injected oocytes in hypotonic medium are plotted in Fig. 1; only 1 min of *Ec*AQP swelling is plotted due to bursting of some oocytes between 1 and 2 min (data not shown). At 1 min, *Ec*AQP oocytes swelled on average 8.0±0.8%, compared with 1.8±0.2% for water-injected control oocytes; at 2 min, control oocytes had only swelled 3.4±0.35%. Endogenously expressed AQP 3 (Schreiber et al., 2000) as well as osmosis across the lipid bilayer may account for swelling of control oocytes. The osmotic permeability, P_f, of *Ec*AQP oocytes, 87 μm/s, differed significantly ($P \ll 0.001$) from that of controls (19 μm/s) (Fig. 1, inset). Osmotic permeability of $HgCl_2$-treated and -untreated *Ec*AQP oocytes did not differ significantly for any of the tested $HgCl_2$ concentrations (Fig. 2). Conductivities of *Ec*AQP oocytes to glycerol ($1\times10^{-4}\pm6\times10^{-5}$ d$(V/V_0)\cdot s^{-1}$) and urea ($3\times10^{-4}\pm6\times10^{-5}$ d$(V/V_0)\cdot s^{-1}$) did not differ significantly from those of control oocytes.

3.2. Sequence analysis and phylogenetic construction

Kyle–Doolittle hydropathy analysis reveals *Ec*AQP to be a highly hydrophobic protein, as would be expected for an aquaporin; six transmembrane segments are predicted (residues 13–37, 42–68, 79–107, 131–157, 180–208 and 222–250). Comparison of *Ec*AQP with existing proteins in the databases using BLAST revealed AQP A of *Dictyostelium discoideum* to be the most similar (26% identity) among named, characterised proteins; among human aquaporins, AQP2 was the closest match (24% identity). In an unrooted phylogenetic construction of *Ec*AQP, human AQPs 0–9, and AQPs from several parasitic protists, two plants, and a yeast (Fig. 4), *Ec*AQP clusters neither with the orthodox AQPs, which conduct only water (AQPs 0, 1, 2, 4–6), nor with the water- and solute-conducting aquaglyceroporins (AQPs 3, 7 and 9). Instead it branches closely with two other protist AQPs, one of which is an orthodox AQP of *Trypanosoma cruzi* (Montalvetti et al., 2004) while the other is an aquaglyceroporin of *Toxoplasma gondii* (Pavlovic-Djuranovic et al., 2003), and with an orthodox AQP from the yeast *Saccharomyces cerevisiae* (Laize et al., 2000). Alignment of *Ec*AQP with human AQP 1 (Fig. 4) demonstrates the presence within *Ec*AQP of the two NPA motifs believed to line the AQP aqueous pore (Jung et al., 1994), and the absence of the cysteine residue aligned with C189 of AQP 1, which is believed to confer mercury-sensitivity to AQP 1 (Preston et al., 1993).

4. Discussion

Hundreds of AQPs or putative AQPs have been identified, from each of the three domains of life. Aquaporins are thought to exist natively as a homotetramer, with each 26–34 kDa monomer forming its own pore (Verkman and Mitra, 2000). The expected molecular weight of the *Ec*AQP protein is approximately 26.8 kDa; thus it is within the range for an AQP monomer. An `hourglass model' has been posited for the shape of the monomer (Jung et al., 1994), in which six transmembrane domains surround the pore, formed in part by two NPA motifs. These characteristics are also predicted for *Ec*AQP (Fig. 4). In addition, the BLAST similarity searches strongly suggest that *Ec*AQP is a member of the AQP protein family.

The significantly increased permeability of *Ec*AQP-injected *Xenopus* oocytes as compared to controls (Fig. 1, inset) provides further evidence that the AQP-like gene within the *E. cuniculi* genome (Katinka et al., 2001) is indeed an aquaporin. Aquaporins of selected other protistan parasites have measured P_f s of 32 (*T. cruzi*; (Montalvetti et al., 2004), 40 (*T. gondii*;(Pavlovic-Djuranovic et al., 2003), and 276 μm/s (*Plasmodium falciparum*;(Hansen et al., 2002). The *Ec*AQP P_f (87 μm/s) is similar to those of human AQPs 3, 2, and 5 (80, 100 and 100 μm/s, respectively, (Yang and Verkman, 1997), which are considered to be in

the high range for human aquaporins (King et al., 2004). However, because of differing levels of expression attributable to, among other factors, different amounts of cRNA injected by each investigator, and their varying translational and post-translational processing efficiencies, these comparisons are not strictly quantitative.

Aquaporins are divided into two phylogenetically and functionally distinct groups (reviewed in Heymann and Engel, 1999): the classic, or orthodox AQPs, which are permeable to water, and the aquaglyceroporins, which are also permeable to glycerol and other small solutes. Neither the presence nor absence of solute conductivity by *Ec*AQP can be predicted based on the phylogeny in Fig. 3, as it clusters with neither group among the human AQPs, and branches closely with non-human AQPs of both types. However, its lack of solute conductivity, 3×10^{-4} $d(V/V_0)\cdot s^{-1}$, not significant relative to water-injected controls, and approximately an order of magnitude lower than the reported swelling rates of $\sim2\times10^{-3}$ $d(V/V_0)\cdot s^{-1}$ for the *T. gondii* (Pavlovic-Djuranovic et al., 2003) and *P. falciparum* aquaglyceroporins (Hansen et al., 2002), is perhaps not surprising in light of the fact that *Ec*AQP shares highest identity (24%) among the human AQPs with orthodox AQP 2. *Ec*AQP also branches closely with a yeast AQP (S. cerevisiae AQP 2; (Laize et al., 2000) (Fig. 3), which is consistent with recent data on the fungal origins of the phylum Microsporidia (Thomarat et al., 2004).

Mercury-inhibition of osmotic permeability is a hallmark of many aquaporins (Yang, 2000) and the observation that germination of spores of the microsporidian *B. algerae* was inhibited by treatment with mercury salts (Frixione et al., 1997) was interpreted as circumstantial evidence for microsporidian AQPs. It is also possible, however, that the inhibitory effect of mercury on germination observed by Frixione et al. (1997) is attributable to modification of other cysteine-containing micro-sporidian proteins. For example, Hayman et al. (2001) identified two *Encephalitozoon intestinalis* spore proteins with N-terminal cysteine-rich motifs, whose functions are as yet unknown. General cytotoxic effects of mercury may also be partially or wholly responsible for the observed inhibition of germination.

Pre-treatment of *Ec*AQP-expressing oocytes with $HgCl_2$ did not inhibit swelling. Nonetheless, this does not preclude the classification of *Ec*AQP as an AQP, as several AQPs have documented mercury-insensitivity, e.g. the prototypical mercurial-insensitive AQP 4 (Yang et al., 1995). Cysteine residue 189 (C189), which is close in primary sequence to the second of the NPA motifs which have been postulated to line the aqueous pore (Jung et al., 1994), has been shown by site-directed mutagenesis to be the mercury-sensitive residue of AQP 1 (Preston et al., 1993) and site-directed mutagenesis to cysteine of any of four amino acids near the first NPA motif or one amino acid near the second NPA was found to confer mercury-sensitivity to AQP4 of *Rattus norvegicus* (Shi and Verkman, 1996). Alignment of *Ec*AQP and AQP 1 amino acid sequences (Fig. 4) demonstrates that the *Ec*AQP amino acid near the second NPA corresponding to the C189 of AQP 1 is glycine (i.e. *Ec*AQP G203), as well as a lack of any cysteine residues in the immediate vicinity of the first NPA motif. The absence of these cysteines may explain the apparent mercury-insensitivity of *Ec*AQP. Among human AQPs, the mercurial-insensitive AQP 4 is the second-closest match (22% identity) to *Ec*AQP according to BLAST analysis. In the future, it may be interesting to examine whether mercury-sensitivity could be conferred upon *Ec*AQP by site-directed mutagenesis of G203 to a cysteine residue.

In conclusion, we believe that the functional swelling assay in *Xenopus* oocytes and amino acid sequence analysis provide evidence that the putative AQP-like sequence identified in the *E. cuniculi* genome (Katinka et al., 2001) may indeed be considered an AQP. As the germination of microsporidian spores is believed to depend on the rapid influx of water

(reviewed in Keohane and Weiss, 1999), it is hoped that further study of microsporidian AQPs and potential inhibitors of these proteins (e.g. gold and silver salts (Niemietz and Tyerman, 2002)) may yield novel therapeutic agents for human infections with these opportunistic pathogens.

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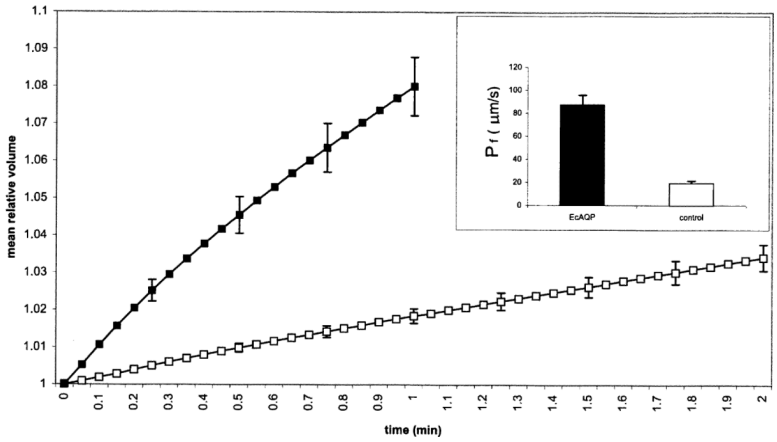

Fig. 1.

*Ec*AQP-injected *Xenopus laevis* oocyte swelling assay. After 1 min, *Ec*AQP-injected oocytes had swelled an average of 8.0 vs. 1.8% water-injected controls. Inset: Altered water permeability ($P \ll 0.001$) of *Ec*AQP-injected oocytes (87.3±8.6 μm/s; $n=12$) vs. water-injected (19.3±2.0 μm/s; $n=15$). Legend: closed marker/bar, *Ec*AQP-injected; open marker/bar, water-injected. (Mean±S.E.M; for clarity, error bars are only displayed every 15 s.).

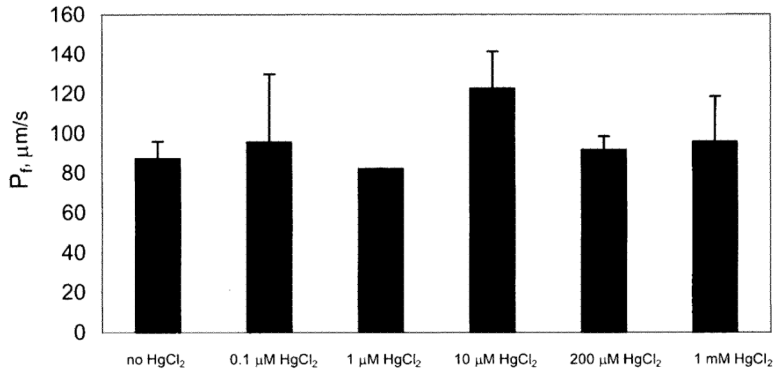

Fig. 2.

Swelling of *Ec*AQP oocytes is not inhibited by H_gCl_2. Permeability differences between H_gCl_2-treated and -untreated oocytes were not significant (where $n>1$) for all tested concentrations (0.1 µM, $n=2$; 1 µM, $n=1$; 10 µM, $n=5$; 200 µM, $n=4$; 1 mM, $n=2$; untreated, $n=12$).

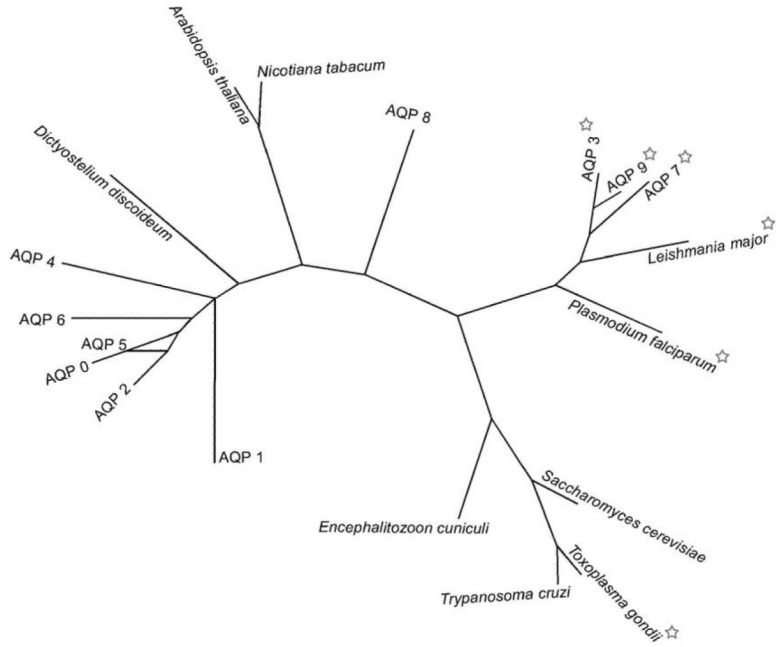

Fig. 3.

An unrooted phylogenetic tree of aquaporins (AQP) including *Ec*AQP. This is based on CTREE alignment of protein sequences of *Ec*AQP (GenBank accession no. NP_586002), closest BLAST match AQP A of *Dictyostelium discoideum* (BAA85158), human AQPs 0-9 (NP_036196, NP_932766, NP_000477, NP_004916, P55087, NP_001642, Q13520, NP_001161, O94778, NP_066190, respectively), plant aquaporins of *Arabidopsis thaliana* (P25818) and *Nicotiana tabacum* (CAA69353), parasitic protist aquaporins of *Leishmania major* (AAS73184), *Plasmodium falciparum* (CAC88373), *Toxoplasma gondii* (CAE46485), *Trypanosoma cruzi* (AAM76680), and AQP 2 of the yeast *Saccharomyces cerevisiae* (AAD10058). Stars indicate aquaglyceroporins.

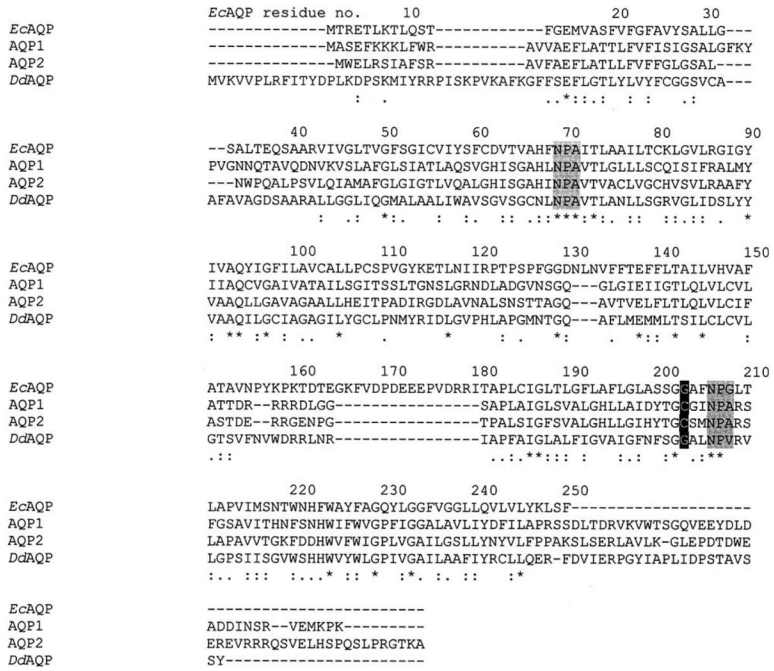

Fig. 4.

Alignment of *Ec*AQP, human AQPs 1 (accession no. NP_932766), 2 (NP_000477), and AQP A of *Dictyostelium discoideum* (BAA85158). Highlighted in black is the residue position at which the presence of a cysteine confers mercury-sensitivity to AQP 1; gray highlights are the NPA motifs thought to line the water-conducting pore of each AQP monomer. Asterisks indicate fully conserved residues; two dots, conservation of strong groups; one dot, conservation of weak groups.