Molecular cloning and characterization of *Echinostoma caproni* heat shock protein-70 and differential expression in the parasite derived from low- and high-compatible hosts

M. HIGÓN[1], C. MONTEAGUDO[2], B. FRIED[3], J. G. ESTEBAN[1], R. TOLEDO[1] *and* A. MARCILLA[1]*

[1] *Departamento de Biología Celular y Parasitología, Facultat de Farmàcia, Universitat de Valencia, Av. V.A. Estellés, s/n, 46100 Burjassot, Valencia, Spain*
[2] *Departamento de Patología, Facultat de Medicina, Av. Blasco Ibañez, 17, Valencia, Spain*
[3] *Department of Biology, Lafayette College, Easton, Pennsylvania 18042, USA*

(*Received 29 April 2008; revised 18 July 2008; accepted 18 July 2008; first published online 28 August 2008*)

SUMMARY

We cloned and expressed *Echinostoma caproni* HSP70 in *Escherichia coli*. This molecule presents an open reading frame (ORF) of 655 amino acids, and a theoretical molecular weight of 71 kDa. *E. caproni* HSP70 protein showed a high homology to other helminth molecules, major differences being located in the C-terminal region of the molecule, with a hydrophobic portion. Studies of protein and messenger RNA (mRNA) expression revealed a distinct pattern, depending on the host (low- or high-compatible). Specific polyclonal antisera raised against the recombinant protein expressed in *Escherichia coli* demonstrated its selective presence in excretory/secretory products (ESP) of adult parasites obtained from high-compatible hosts. Immunological studies showed clearly the association of HSP70 with the parasite surface and other structures, including eggs.

Key words: *Echinostoma caproni*, heat shock protein, HSP70, molecular cloning and expression, immunolocalization, parasite, trematode.

INTRODUCTION

The heat shock protein-70 (HSP70) family constitutes a group of conserved proteins acting as molecular chaperones which fold, assemble and translocate other proteins (Robert, 2003; Mayer and Bukau, 2005). Their widespread expression in different organisms have made HSP70s useful for various purposes, including phylogenetic studies (Borchiellini *et al*. 1998), as well as representing a biomarker for monitoring environmental pollution (Arts *et al*. 2004). These proteins are critical for parasite survival (Polla, 1991; Thompson *et al*. 2008). Several studies have identified HSP70s as major immunogens in parasitic infections, either through the analysis of humoral responses in the host (Moser *et al*. 1990; Ortona *et al*. 2003; Sotillo *et al*. 2008), the screening of recombinant expression libraries using sera from hosts infected with distinct helminths (Colebrook and Lightowlers, 1997; Scott and McManus, 1999; Kanamura *et al*. 2002; Ravi *et al*. 2004) or immunochemical studies (Vayssier *et al*. 1999). Recent studies describing the knockdown of HSP70 in *Caenorhabditis elegans* have indicated a potential role for HSP70 as a target for parasite intervention (Kimura *et al*. 2007), confirming a previous hypothesis regarding its function in cell death in the parasitic nematode *Trichinella spiralis* (see Martinez *et al*. 2004).

The course and characteristics of *Echinostoma caproni* infection are largely dependent on host-related factors (Toledo and Fried, 2005). This knowledge has allowed workers to define hosts with high or low compatibility for several echinostome species. The differences between host species with varied compatibility are reflected predominantly in worm establishment and growth, egg output, parasite fecundity and survival of the worms. In high-compatible hosts, such as mice and hamsters, *E. caproni* infection becomes chronic, and the parasite can survive for up to 20 weeks, whereas the worms are rapidly expelled in low-compatible hosts, such as rats (Toledo and Fried, 2005). Our research group has recently identified HSP70 in excretory/secretory products (ESP) from *Echinostoma* spp. (Bernal *et al*. 2006; Sotillo *et al*. 2008). Interestingly, this molecule was more abundant in *E. friedi* ESP obtained from high-compatible than from

* Corresponding author: Departamento de Biología Celular y Parasitología, Facultat de Farmàcia, Universitat de Valencia, Av. V.A. Estellés, s/n, 46100 Burjassot, Valencia, Spain. Tel: +34 963544491. Fax: +34 963544769. E-mail: antonio.marcilla@uv.es

doi:10.1017/S0031182008004927 Printed in the United Kingdom

low-compatible hosts, suggesting a possible role of HSP70 in inflammatory responses (Bernal *et al*. 2006). Also, a recent study has revealed that variation in HSP70 gene expression in *Fasciola* species are also related with host compatibility (Smith *et al*. 2008).

In spite of HSP70s being predominantly intracellular proteins, there is increasing evidence for their expression on the cell surface in helminths. A study of *Brugia malayi* has detected HSP70 in musculature, hypodermis and reproductive organs (Schmitz *et al*. 1996), and a recent report described it as one of the most abundant surface antigens in *Schistosoma mansoni* egg secretions based on proteomic analysis (Cass *et al*. 2007).

In the present study, we cloned *E. caproni* HSP70 (*Ec*HSP70) and immunolocalized this molecule in adult parasites using polyclonal antibodies raised against the recombinant protein produced in *Escherichia coli*.

MATERIALS AND METHODS

Parasites and experimental infections

The techniques used for the maintenance of *Echinostoma caproni* in the laboratory have been described (Toledo *et al*. 2004). Encysted metacercariae of *E. caproni* were removed from the kidneys and pericardial cavities of experimentally infected snails (*Biomphalaria glabrata*) and used to infect golden hamsters (*Mesocricetus auratus*) as well as albino Wistar rats (*Rattus norvegicus*). Outbred male golden hamsters, weighing 45–60 g, and male rats, weighing 110–150 g, were each infected (via gavage) with 100 metacercariae of *E. caproni*. The rats were maintained under standard conditions and provided with food and water *ad libitum*. The number of *E. caproni* eggs released per rat per day was recorded, as described previously (Toledo *et al*. 2003).

Parasite antigens

Excretory/secretory products (ESP) and somatic lysates were obtained as reported previously (Marcilla *et al*. 2007). Tegumentary proteins were obtained using Triton X-100, following the protocol by Anuracpreeda *et al*. (2006). Identical weights of *E. caproni* adults from rats and from hamsters were washed twice with 10 mM Tris-HCl, pH 7·4, 150 mM NaCl (TBS), and treated with 1% Triton-X100 in 50 mM Tris-HCl buffer, pH 8·0, 10 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, for 20 min at room temperature. The integrity of the parasites was monitored by microscopical examination. Soluble tegumentary material was obtained following centrifugation at 10 000 ***g*** for 20 min. The protein content of individual samples was determined using the Bradford method (Bio-Rad).

Cloning of full-length EcHSP70 gene

Total RNA from *E. caproni* obtained from hamsters and rats was isolated using the Rapid Total RNA purification system (Marligen Biosciences Inc.), according to the manufacturer's instructions. The cDNA was synthesized using the SuperScript™ III system employing the oligo(dT)12–18 primer supplied (Invitrogen). Internal primers to the HSP70 gene were designed, aligning the helminth HSP70 sequences available in the databases (*Caenorhabditis elegans*, M18540; *Onchocerca volvulus*, J04006, *Echinococcus granulosus*, V26448; *Schistosoma mansoni*, X05384 and *Schistosoma japonicum*, AF044412) using the online Clustal W 1.8 program (www.ebi.uk), and choosing 2 conserved regions (corresponding to regions 343–350 and 454–461 of the *S. mansoni* molecule). All oligonucleotides used in the present study are listed in Table 1 as Supplementary material. The polymerase chain reaction (PCR) was performed using 2 μl of cDNA employing the following cycling protocol: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 50 s; and 72 °C for 5 min in an MJ Research MiniCycler (MJ Research).

To obtain a full-length copy of *EcHSP70*, 5′ and 3′ rapid amplification of cDNA ends (RACE) by PCR was performed using a SMART RACE cDNA Amplification kit® (Clontech), according to the manufacturer's instructions. The 5′ RACE technique was used to generate (full-length) cDNA in the 5′ direction using 5′ RACE system. First-strand cDNA synthesis was initiated on total RNA (0·6 μg) using the oligo(dT) anchor primer (5′ CDS RACE) and the SMART primer. The 5′ RACE-PCR product was amplified using the 5′ RACE adaptor primer (UPM, universal primer mix) and GSP1. The 3′ RACE was used to generate full-length cDNA in the 3′ direction using 3′ RACE system. First-strand cDNA synthesis was initiated at the total RNA (0·6 μg) using the oligo(dT) anchor primer (3′ CDS RACE). The 3′ RACE product was amplified by PCR using primer GSP2 and the 3′ RACE adaptor primer (UPM). These two PCRs were performed under the following conditions: 2·5 μl (~0·2 μg) of cDNA, 10 μM primer GSP1 (or GSP2) and UPM, 10 mM of each dNTP and TITANIUM™ *Taq*DNApolymerase (5 U/μl, Clontech) in 50 μl of buffer. The PCR was carried out for 26 cycles as follows: 94 °C for 30 s for denaturation, 68 °C for 30 s (annealing) and 72 °C for 3 min (extension).

PCR fragments were subcloned in the pGEM-T™ vector system (Promega Biotech Iberica, Spain) following the manufacturer's instructions and sequencing (both strands) was performed using the *Taq* DyDeoxy terminator method (Applied Biosystems Inc.) and run on an Applied Biosystems 373A DNA sequencer. The analysis of nucleotide and deduced amino acid sequences was conducted

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press

using the ExPASy package of software on line (www.expasy.org). Database comparisons against GenBank were made using the BLAST program (Altschul *et al*. 1997).

Expression of the EcHSP70 in bacteria

A PCR fragment containing the whole *EcHSP70* gene was obtained using the 5′ RACE-PCR product as a template, employing the primers H70FBam (forward), and H70RPst (reverse), which contained the restriction enzyme sites for *Bam*HI and *Pst*I, respectively (Table 1, in Supplementary material), to assist cloning in the expression vector pQE30, which fuses the protein to a polyhistidine peptide in the N-terminal region (Qiagen). The PCR products were digested with *Bam*HI and *Kpn*I, and then cloned into the vector pQE30. *E. coli* M15 strain (Qiagen) was transformed with the recombinant plasmid, and the expression of a polyhistidine-containing recombinant protein was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM at 37 °C for 4 h. The induced cells were harvested and lysed in a buffer containing 8 M urea, 100 mM KH_2PO_4, 10 mM Tris-HCl, pH 8·0. The histidine-tagged proteins were affinity-purified using a His-Trap kit (Qiagen), according to the manufacturer's instructions. The recombinant protein (r*Ec*HSP70) was eluted with 8 M urea, 100 mM KH_2PO_4, 10 mM Tris-HCl, pH 4·5.

Production of anti-EcHSP70 antisera

Polyclonal antisera against r*Ec*HSP70 were obtained by inoculating two 3 kg New Zealand white rabbits with 100 μg of the protein emulsified in 0·3 ml of Freund's complete adjuvant. Rabbits were given similar inoculations of Freund incomplete adjuvant at 5 different time-points (2 weeks apart). Blood was drawn 21 days after the final inoculation. Antibody titres were confirmed by indirect ELISA as described (Toledo *et al*. 2004).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

SDS-PAGE in 10% polyacrylamide gels was performed as described previously (Marcilla *et al*. 1995). Gels were transferred to nitrocellulose membranes in 20 mM Tris, 192 mM glycine, methanol 20% (v/v), pH 8·3. Membranes were stained with 0·1% Ponceau S (Sigma) for 10 min, and blocked for 3 h in TBS containing 5% (w/v) skim milk. After extensive washes of the membranes with TBS containing 0·05% (v/v) Tween-20 (TBST), blots were incubated in TBS + 3% bovine serum albumin (BSA) with anti-r*Ec*HSP70 rabbit antisera. The recombinant protein (r*Ec*HSP70) was used as a control. The bound antibodies were detected by incubating blots for 1 h at room temperature (22–24 °C) with horseradish peroxidase (HRP) conjugated to goat anti-rabbit IgG (Bio-Rad) in TBST-1% (w/v) BSA. After 5 washes with TBST, immune complexes were detected using Lumi-light Western blotting substrate (Roche), following the manufacturer's instructions and images captured with a ChemiDoc™ XRS system (Bio-Rad).

Immunohistochemical detection of EcHSP70 in E. caproni *adults*

The immunolocalization of HSP70 in *E. caproni* adult worms from rats and hamsters was studied using immunohistochemical techniques. Endogenous peroxidase activity was neutralized by incubation in 3% (v/v) H_2O_2 at room temperature for 30 min. The sections were then blocked for 1 h with 1% (w/v) BSA to prevent non-specific binding. Immunohistochemistry was performed on 4 μm thick, paraffin-embedded *E. caproni* sections using the avidin-biotin-immunoperoxidase technique (LSAB, Dakocytomation, Denmark, following the manufacturer's instructions), with diaminobenzidine (Dakocytomation) as the chromogen. Anti-EcHSP70 antiserum, diluted 1 : 200 and incubated for 1 h at room temperature, served as primary antibody. Appropriate controls were included in all assays, with TBS and secondary antibody incubations being used as negative controls.

RESULTS

Cloning and DNA sequence analysis of E. caproni *HSP70*

To clone the *E. caproni* HSP70 gene, degenerate PCR primers were designed based on conserved amino acid sequence motifs present within the HSP70 family of proteins (Supplementary Table 1). The amplification of *E. caproni* cDNA by RT-PCR yielded a single product of 323 bp (data not shown). Sequence analysis of this DNA product revealed unambiguous homology (from 79 to 85%) to the HSP70 superfamily, having the best match (85% nucleotide identity) with the orthologue/homologue from the cestode *Echinococcus granulosus* (see Colebrook and Lightowlers, 1997).

The entire coding sequence for *E. caproni* HSP70 was obtained by 5′ and 3′ RACE-PCR using cDNA as the template. The resultant cDNA was 2040 bp in size with an open reading frame (ORF) of 1968 bp, encoding 655 amino acids with a predicted molecular weight of 71708·94 Da and an isoelectric point of 5·5, as calculated *via* the ExPASy server (Gasteiger *et al*. 2005). The sequence data have been deposited in EMBL nucleotide sequence database (Accession number AM710614). This sequence contained the typical HSP70 signature motifs when compared

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press

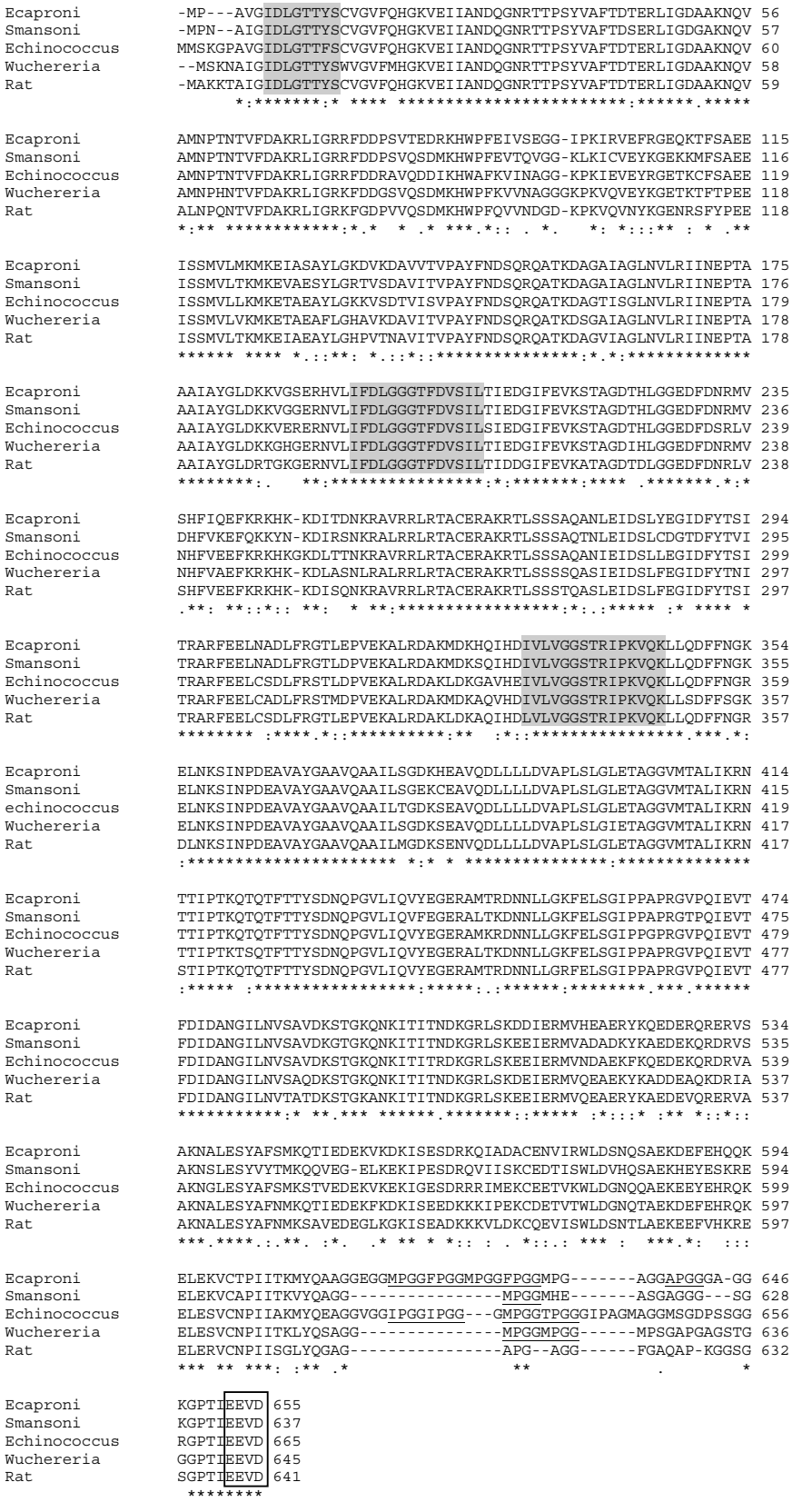

Fig. 1. Amino acid sequence alignment of *Echinostoma caproni* HSP70 (*Ec*HSP70) with various HSP70s from other trematodes, selected nematodes and rat using Clustal W 1.8. Accession numbers of the aligned sequences are the following: *Schistosoma mansoni* (P08418); *Echinococcus granulosus* (AAA99139); *Wuchereria bancrofti* (AAF32254); and rat, *Rattus novergicus* (AAA17441). Amino acid residues identical among all 5 sequences are indicated by asterisks, and conserved and semi-conserved substitutions with dots. Fingerprinting domains for HSP70 (ExPASy) are shadowed, hydrophobic tetrapeptide repeats are underlined, and the ending motif is boxed.

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press

with other HSP70s (Fig. 1). A multiple-alignment of *Ec*HSP70 with other HSP70 sequences available from current databases revealed ~84% homology with helminth proteins (from *Schistosoma mansoni*, *Echinococcus granulosus* and *Wuchereria bancrofti*), and 81% with the molecule from rat, ruling out the possibility of host contamination. Major differences in amino acid sequence were located at the C-terminal region of the HSP70s. *Ec*HSP70 presented the typical HSP70 eukaryotic motifs GGMP and the ending EEVD, but contained 6 repetitive units of the hydrophobic tetrapeptide GGXP (3 of them being GGMP). These units were present in lower numbers in the other helminth sequences examined (see Fig. 1).

Differential expression of EcHSP70 in E. caproni *between low- and high-compatible hosts*

For the analysis of the expression of HSP70 in *E. caproni* between low- and high-compatible hosts, we used a specific rabbit antiserum against the *E. caproni* HSP70 recombinant protein (r*Ec*HSP70) produced in *E. coli*. The serum detected the *Ec*HSP70 in ESP from *E. caproni* which originated from hamster (high-compatible host), but not in material from the same species collected from rat (low-compatible host) (Fig. 2A). The molecule was detected in somatic lysates from *E. caproni* from either host, with a slightly lower amount in parasite material from the low-compatible host (Fig. 2A). To address whether the differences in the amount of HSP70 correlated with gene expression in these hosts, we performed semi-quantitative RT-PCR using mRNA from adult parasites from low- and high-compatible hosts, respectively.

A set of primers was designed to carry out RT-PCR assays (see Table 1 in Supplementary material). Identical RNA amounts were prepared from *E. caproni* adults obtained from rats and hamster (3 weeks after experimental infection). The results indicated that *Ec*HSP70 was differentially expressed between both hosts at that time, with a somewhat higher level of HSP70 mRNA being detected in *E. caproni* from hamsters (high-compatible host) (Fig. 2B, upper panel). Interestingly, the pattern of actin expression contrasted that of HSP70, with more mRNA being transcribed in *E. caproni* adults from rats (low-compatible host) than from hamsters (high-compatible host) (Fig. 2B, lower panel).

Immunolocalization of EcHSP70 in tissue sections of E. caproni *adults from low- and high-compatible hosts*

Subsequently, we studied the localization of the native *Ec*HSP70 to structures in adult *E. caproni*, and compared its presence in this species collected from

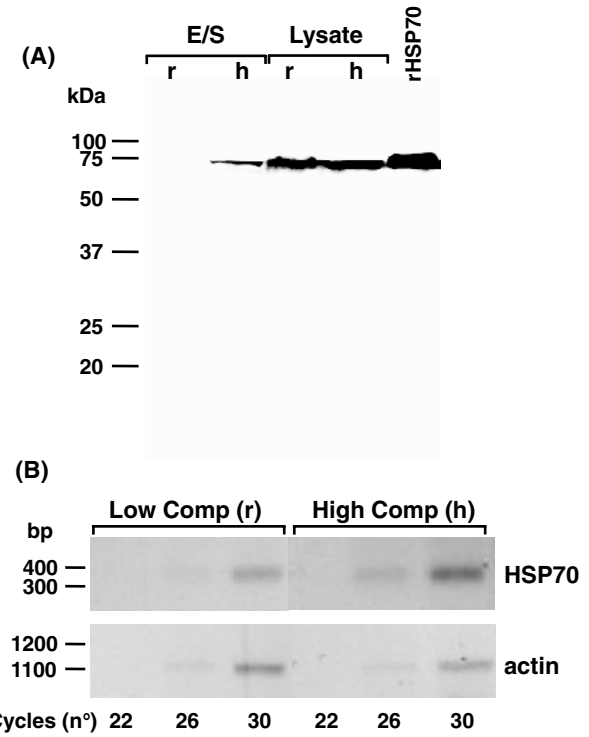

Fig. 2. Analysis of expression of HSP70 from *Echinostoma caproni*. (A) Western-blot of *E. caproni* excretory-secretory products (ESP) and total lysates developed with rabbit antisera obtained against recombinant *Ec*Hsp70 produced in *E. coli* (r*Ec*HSP70). Samples of 100 μg (measured as protein) of ESP and total lysates of *E. caproni* adults collected from rat (low-compatible host, r) or hamster (high-compatible host, h) were analysed. The bacterial r*Ec*HSP70 protein (rHSP70) was used as a positive control. The position of molecular weight markers in kDa is indicated. (B) Semi-quantitative-PCR using mRNA from *E. caproni* adults from low-compatible (rat, r) or high-compatible hosts (hamster, h). The number of PCR cycles and position of DNA markers in bp are indicated.

each host (low- or high-compatible hosts) by means of immunological staining using rabbit antisera anti-r*Ec*HSP70. As shown in Fig. 3A–D, most of the staining was detected in eggs in the uterus of adult worms from both host species. Staining was also observed in the tegument and in the parenchyma, with slight differences between worms from the two hosts (Fig. 3A, B). No staining was observed in controls using only secondary antibodies (data not shown). At high magnification (×250), minor differences were observed in the localization of HSP70 on the worm surface between the two different host species (Fig. 3C, D). Protein *Ec*HSP70 was located at the outer layer of the tegument, and was also detected in the subtegument and parenchyma in adult *E. caproni* from hamsters (high-compatible host) (Fig. 3D).

To confirm the presence of *Ec*Hsp70 in the tegument and compare the amount of this protein in

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press

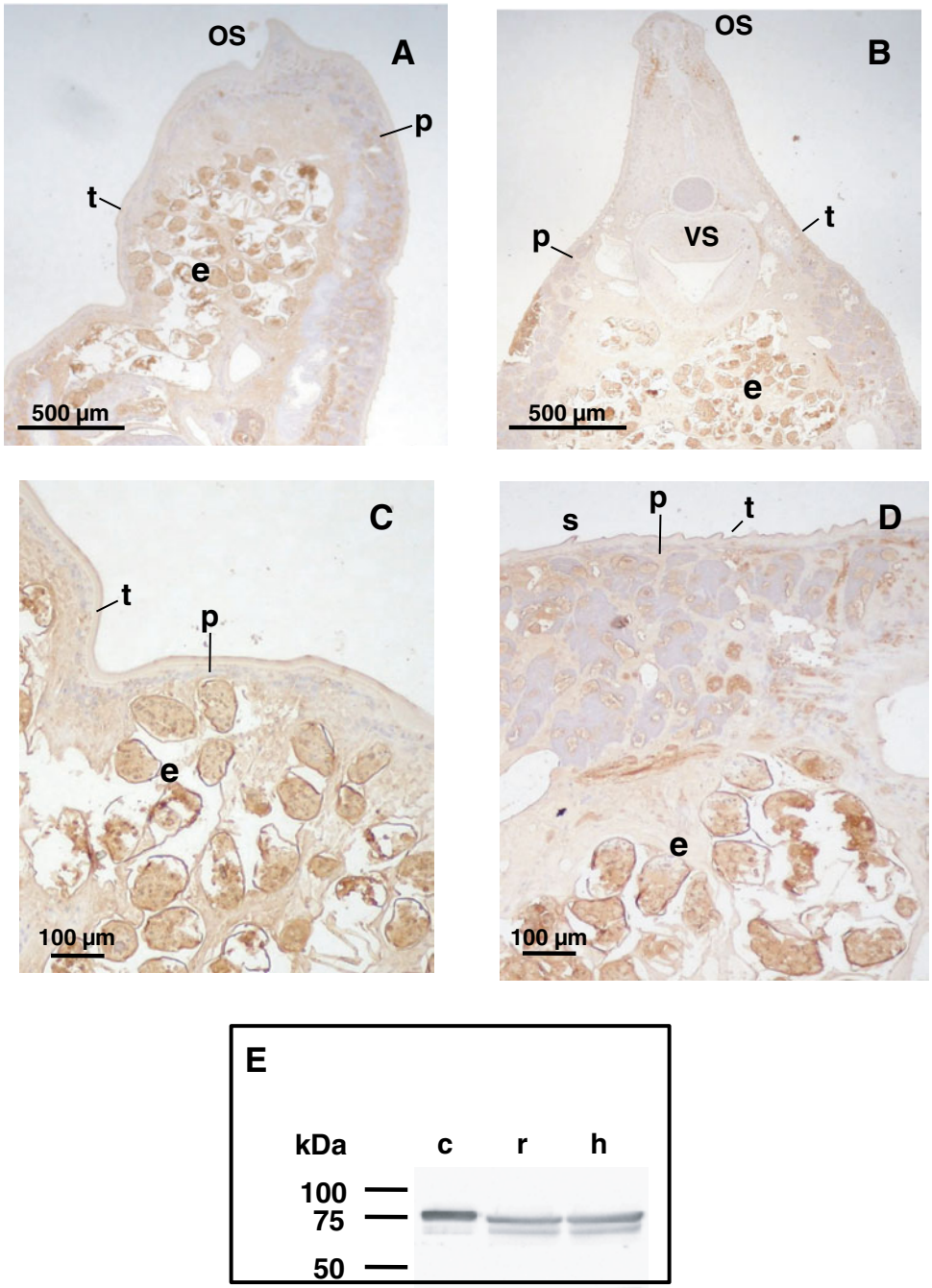

Fig. 3. Localization of HSP70 in adults of *Echinostoma caproni*. (A–D) Immunohistochemical detection of *Ec*HSP70 in tissue sections of adult *E. caproni* by rabbit anti-r*Ec*HSP70, (×100). (A) Sagittal section of 3-week-old adult obtained from rat, (×100). (B) Frontal section of 3-week-old adult obtained from hamster. (C) High magnification (×250) of (A), detailing reactivity of eggs and tegument. (D) High magnification (×250) of (B), detailing tegumentary and subtegumentary reactivity. Os, oral sucker; vs, ventral sucker; s, spine; e, eggs; t, tegument; p, parenchyma. (E) Western-blot of adult *E. caproni* tegumentary proteins using anti-r*Ec*HSP70 antiserum. Tegumentary proteins (25 µg/lane) obtained from *E. caproni* adults collected either from rat (r) or hamster (h). r*Ec*HSP70 protein (c) was used as a control. The position of molecular weight markers in kDa is indicated.

worms from rats (low-compatible host) or hamsters (high compatible host), adults from each host were subjected to tegumentary stripping using a brief treatment with detergent (Triton X-100) (see Anuracpreeda *et al*. 2006). As shown in Fig. 3E, *Ec*Hsp70 was detected in the tegumental material by immunoblot analysis, with no appreciable variation in the amount of *Ec*Hsp70 between the two samples derived from the different hosts (Fig. 3E).

DISCUSSION

In the present study, we cloned HSP70 from *E. caproni* and expressed it as a recombinant

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press

molecule. It has an estimated molecular weight of 71 kDa, and the predicted amino acid sequence shows many similarities to eukaryotic HSP70s, including the repetitive sequence GGMP at the C-terminal region, which has been linked to HSP70 functionality (Demand *et al*. 1998). This tetrapeptide has been described in other parasitic helminths, such as *W. bancrofti* (see Ravi *et al*. 2004), *Brugia malayi* (see Rothstein and Rajan, 1991), *S. japonicum* and *S. mansoni* (see Hedstrom *et al*. 1988) as well as in *Plasmodium falciparum* (see Kumar *et al*. 1990) and the protozoan parasite *Trypanosoma cruzi* (see Engman *et al*. 1990). The presence of this GGMP peptide has been associated with the immunological properties of HSP70 in *P. falciparum* (see Richman and Reese, 1988; Kumar *et al*. 1990), as well as with differences in virulence detected in different strains of *Toxoplasma gondii* (reviewed by Miller *et al*. 1999). In *E. caproni*, this GGMP sequence is repeated 3 times, alternating with 2 repeats of the related sequence GGFP, comprising a relatively large hydrophobic region. This latter characteristic could account for the external localization of HSP70 on *E. caproni*, in contrast to related molecules of *Fasciola hepatica* and *F. gigantica*, which are not detected in ESP and seem to contain only 1 tetrapeptide GGMP (Smith *et al*. 2008).

*Ec*HSP70 is present on the surface of *E. caproni* as well as in eggs. These findings seem similar to those for HSP70 of *Brugia malayi*, being present in the somatic musculature, hypodermis and reproductive structures (Schmitz *et al*. 1996) and also support the recent finding that HSP70 is one of the most abundant proteins in *S. mansoni* egg secretome (Cass *et al*. 2007). The amount of *Ec*Hsp70 detected by immunoblot on the surface of adults of *E. caproni* from the low-compatible host (rat) does not differ appreciably from that in parasites from the high-compatible host (hamster). This information suggests that there is no direct relationship between amount in the tegument and that present in excretory/secretory products (ESP), as *Ec*Hsp70 was absent from ESP from *E. caproni* from the low-compatible host.

The present results demonstrated a higher level of transcription and also HSP70 secretion from *E. caproni* from the high-compatible host (hamster) compared with the low-compatible host (rat). Since no differences were observed in the amount of *Ec*Hsp70 either in somatic lysates or surface materials, the increase in transcription in *E. caproni* from hamsters could correlate with a higher secretion of the molecule, which in turn could facilitate the parasite establishment in the host, and/or modulate the host response. In support of this notion, we have previously shown an enrichment of parasite ESP in the intestinal mucosa in the host as well as mucosal damage caused by *E. caproni* in hamsters (high-compatible hosts) (Toledo *et al*. 2006). More recent studies have revealed that increased levels of HSP70 may account for virulence of parasites by adapting to the host environmental changes and modulating host immune responses (see Song *et al*. 2007). Recently, differential expression of HSP70 has also been observed in the liver flukes *Fasciola hepatica* and *F. gigantica*, although the molecule(s) were not detected in ESP (Smith *et al*. 2008). Intriguingly, the distinct results between species of trematode could be due to differences in life cycle (i.e. with no tissue migration in the *Echinostoma* spp.), and/or parasite localization in the host (biliary ducts for species of *Fasciola versus* the intestine for *E. caproni*).

This work was supported by grants CGL2005-02321/BOS from the Spanish Ministry of Education and Science (Madrid, Spain) and FEDER; GV07/006 y ACOMP07/071 from Conselleria d'Empresa, Universitat i Ciència de la Generalitat Valenciana (Valencia, Spain); and 20050201 (Accions Especials) from the Universitat de València (Valencia, Spain). M.H. is a pre-doctoral fellow (FPU) from the Spanish Ministry of Education and Science.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Anuracpreeda, P., Wanichanon, C., Chaithirayanon, K., Preyavichyapugdee, N. and Sobhon, P. (2006). Distribution of 28·5 kDa antigen in the tegument of adult *Fasciola gigantica*. *Acta Tropica* **100**, 31–40.

Arts, M. J., Schill, R. O., Knigge, T., Eckwert, H., Kammenga, J. E. and Köhler, H. R. (2004). Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. *Ecotoxicology* **13**, 739–755.

Bernal, D., Carpena, I., Espert, A. M., De la Rubia, J. E., Esteban, J. G., Toledo, R. and Marcilla, A. (2006). Identification of proteins in excretory/secretory extracts of *Echinostoma friedi* (Trematoda) from chronic and acute infections. *Proteomics* **6**, 2835–2843.

Borchiellini, C., Boury-Esnault, N., Vacelet, J. and Le Parco, Y. (1998). Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. *Molecular Biology and Evolution* **15**, 647–655.

Cass, C. L., Johnson, J. R., Califf, L. L., Xub, T., Hernandez, H. J., Stadecker, M. J., Yates, J. R. and Williams, D. L. (2007). Proteomic analysis of *Schistosoma mansoni* egg secretions. *Molecular and Biochemical Parasitology* **155**, 84–93.

Colebrook, A. L. and Lightowlers, M. W. (1997). Serological reactivity to heat shock protein 70 in patients with hydatidic disease. *Parasite Immunology* **19**, 41–46.

Demand, J., Lüders, J. and Höfeld, J. (1998). The carboxy terminal domains of Hsc 70 provides binding sites for a distinct set of chaperone cofactors. *Molecular and Cellular Biology* **18**, 2023–2028.

Engman, D. M., Dragon, E. A. and Donelson, J. E. (1990). Human humoral immunity to HSP70 during *Trypanosoma cruzi* infection. *Journal of Immunology* **144**, 3987–3991.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M., Appel, R. and Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The Proteomics Protocols Handbook* (ed. Walker, J. M.), pp. 571–608. Humana Press, Totowa, New Jersey, USA.

Hedstrom, R., Culpepper, J., Schinski, V., Arabian, N. and Newport, G. (1988). Schistosome heat shock proteins are immunologically distinct host like antigen. *Molecular and Biochemical Parasitology* **29**, 275–282.

Kanamura, H. Y., Hancock, K., Rodrigues, V. and Damian, R. T. (2002). *Schistosoma mansoni* heat shock protein 70 elicits an early humoral immune response in *S. mansoni* infected baboons. *Memorias do Instituto Oswaldo Cruz* **97**, 711–716.

Kimura, K., Tanaka, N., Nakamura, N., Takano, S. and Ohkuma, S. (2007). Knockdown of mitochondrial heat shock protein 70 promotes progeria-like phenotypes in *Caenorhabditis elegans*. *Journal of Biological Chemistry* **282**, 5910–5918.

Kumar, N., Zhao, Y., Graves, P., Folgar, J. P., Maloy, L. and Zheng, H. (1990). Human immune response directed against *Plasmodium falciparum* heat shock related proteins. *Infection and Immunity* **58**, 1408–1414.

Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A. and Robbins, K. C. (1995). Identification of the major tyrosine kinase substrate in signaling complexes formed after engagement of Fc gamma receptors. *Journal of Biological Chemistry* **270**, 9115–9120.

Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J. G. and Toledo, R. (2007). *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. *Experimental Parasitology* **117**, 57–64.

Martinez, J., Pérez-Serrano, J., Bernardina, W. E., Rincón, I. and Rodriguez-Caabeiro, F. (2004). Heat shock protein synthesis over time in infective *Trichinella spiralis* larvae raised in suboptimal culture conditions. *Journal of Helminthology* **78**, 243–247.

Mayer, M. P. and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**, 670–684.

Miller, C. D. M., Smith, N. C. and Johnson, A. M. (1999). Cytokines, nitric oxide, heat shock proteins and virulence in *Toxoplasma*. *Parasitology Today* **15**, 418–422.

Moser, D., Doumbo, O. and Klinkert, M. Q. (1990). The humoral response to heat shock protein 70 in human and murine schistosomiasis. *Parasite Immunology* **12**, 341–352.

Ortona, E., Marguti, P., Delunardo, F., Vaccari, S., Riganó, R., Profumo, E., Buttari, B., Teggi, A. and Siracusano, A. (2003). Molecular and immunological characterization of the C-terminal region of a new *Echinococcus granulosus* heat shock protein 70. *Parasite Immunology* **25**, 119–126.

Polla, B. S. (1991). Heat shock proteins in host-parasite interactions. In *Immunoparasitology Today* (ed. Ash, C. and Gallagher, J. B.), pp. A38–A41. Cambridge Elsevier Trends Journals, Cambridge, UK.

Ravi, V., Kubofcki, J., Bandopathyaya, S., Geetha, M., Narayanan, R. B., Nutman, T. B. and Kaliraj, P. (2004). *Wuchereria bancrofti*: cloning and characterization of heat shock protein 70 from the human lymphatic filarial parasite. *Experimental Parasitology* **106**, 1–10.

Richman, S. J. and Reese, R. T. (1988). Immunologic modeling of a 75 kDa malarial protein with carrier-free synthetic peptides. *Proceedings of the National Academy of Sciences, USA* **85**, 1662–1666.

Robert, J. (2003). Evolution of heat shock protein and immunity. *Developmental and Comparative Immunology* **27**, 449–464.

Rothstein, N. M. and Rajan, T. V. (1991). Characterization of an HSP70 gene from the human filarial parasite *Brugia malayi* (nematoda). *Molecular and Biochemical Parasitology* **49**, 229–238.

Schmitz, K. A., Hale, T. J., Rajan, T. V. and Yates, J. A. (1996). Localization of paramyosin, myosin, and a heat shock protein 70 in larval an adult *Brugia malayi*. *Journal of Parasitology* **82**, 367–370.

Scott, J. C. and McManus, D. P. (1999). Identification of novel 70-kDa heat shock protein-encoding cDNA from *Schistosoma japonicum*. *International Journal for Parasitology* **29**, 437–444.

Smith, R. E., Spithill, T. W., Pike, R. N., Meeusen, E. N. T. and Piedrafita, D. (2008). *Fasciola hepatica* and *Fasciola gigantica*: Cloning and characterization of 70 kDa heat-shock proteins reveals variation in HSP70 gene expression between parasite species recovered from sheep. *Experimental Parasitology* **118**, 536–542.

Song, K. J., Song, K. H., Na, B. K., Kim, J. H., Kwon, D., Park, S., Pak, J. H., Im, K. I. and Shin, H. J. (2007). Molecular cloning and characterization of a cytosolic heat shock protein 70 from *Naegleria fowleri*. *Parasitology Research* **100**, 1073–1089.

Sotillo, J., Valero, L., Sánchez del Pino, M. M., Fried, B., Esteban, J. G., Marcilla, A. and Toledo, R. (2008). Identification of antigenic proteins from *Echinostoma caproni* (Trematoda) recognized by mouse immunoglobulins M, A and G using an immunoproteomic approach. *Parasite Immunology* **30**, 271–279.

Thompson, F. J., Barker, G. L. A., Hughes, L. and Viney, M. E. (2008). Genes important in the parasitic life of the nematode *Strongyloides ratti*. *Molecular and Biochemical Parasitology* **158**, 112–119.

Toledo, R., Espert, A., Carpena, I., Muñoz-Antoli, C. and Esteban, J. G. (2003). An experimental study of the reproductive success of *Echinostoma friedi* (Trematoda: Echinostomatidae) in the golden hamster. *Parasitology* **126**, 433–441.

Toledo, R., Espert, A., Carpena, I., Muñoz-Antoli, C., Fried, B. and Esteban, J. G. (2004). Comparative development of *Echinostoma caproni* (Trematoda: Echinostomatidae) adults in experimentally infected hamsters and rats. *Parasitology Research* **93**, 439–444.

Toledo, R. and Fried, B. (2005). Echinostomes as experimental models in adult parasite-vertebrate host interactions. *Trends in Parasitology* **21**, 251–254.

Toledo, R., Monteagudo, C., Espert, C., Fried, B., Esteban, J. G. and Marcilla, A. (2006). *Echinostoma caproni*: Intestinal pathology in the golden hamster, a highly compatible host, and the Wistar rat, a less compatible host. *Experimental Parasitology* **112**, 164–171.

Vayssier, M., Le Guerhier, .F, Fabien, J. F., Philippe, H., Vallet, C., Ortega-Pierres, G., Soule, C., Perret, C., Liu, M., Vega-Lopez, M. and Boireau, P. (1999). Cloning and analysis of a *Trichinella britovi* gene encoding a cytoplasmic heat shock protein of 72 kDa. *Parasitology* **119**, 81–93.

https://doi.org/10.1017/S0031182008004927 Published online by Cambridge University Press