

# Comparative analysis of the *14-3-3* gene and its expression in *Echinococcus granulosus* and *Echinococcus multilocularis* metacestodes

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## SUMMARY

It was suggested that the unlimited proliferative capacity of the *Echinococcus multilocularis* metacestode may be related to overproduction of the 14-3-3 protein. As is known, the proliferative capacities of *E. granulosus* and *E. multilocularis* metacestodes are very different. By comparing the expression levels of the *14-3-3* gene between *in vitro*-obtained *E. granulosus* and *E. multilocularis* metacestodes, we were able to provide experimental evidence of the potential relation between 14-3-3 over-expression and tumour-like growth in *E. multilocularis* metacestodes. RT-PCR and Northern blot experiments indicated that 14-3-3 expression level is about 4-fold higher in the *E. multilocularis* metacestode. This differential expression was confirmed both by immunoblotting and immunocytochemistry experiments, which allowed detection of the protein in the cyst wall from *E. multilocularis* but not in the cyst wall from *E. granulosus*. The alignment of the *Echinococcus 14-3-3* cDNA sequence with known 14-3-3 isoforms from other organisms, grouped the parasite sequence into the tumour growth-related isoforms.† The known relation between over-expression of some 14-3-3 isoforms and tumour-related processes, together with the present results, suggest that the *Echinococcus 14-3-3* protein could be one of the molecules responsible for the differences between *E. granulosus* and *E. multilocularis* metacestode growth behaviour.

Key words: *Echinococcus granulosus*, *Echinococcus multilocularis*, metacestode, *14-3-3* gene, 14-3-3 protein, expression level.

## INTRODUCTION

The two best-known *Echinococcus* species, *E. granulosus* and *E. multilocularis*, produce unilocular (UE) and alveolar (AE) echinococcosis, caused by infection with the metacestode (larval) stage of the parasite. The differences between *E. granulosus* and *E. multilocularis* regarding their metacestode development is a well-known phenomenon (Thompson & Lymbery, 1995). The metacestode of *E. granulosus* is typically unilocular, fluid-filled, consisting of a germinal layer supported externally by a non-cellular laminated layer and surrounded by a host-produced fibrous adventitial layer. In contrast, the metacestode of *E. multilocularis* is a multivesicular, infiltrating structure with no limiting host-tissue barrier, forming a network of filamentous cellular protrusions of the germinal layer, which are responsible for the tumour-like growth of the parasite in human patients.

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† Nucleotide sequence data reported in this paper are available in the EMBL, GenBank™ and DDJB databases under the accession numbers AF207904 and AF207905.

UE and AE are severe parasitic diseases in man. Thus, major efforts have been made towards the improvement of diagnosis, prevention and treatment of echinococcal disease. In the last few years, efforts have been focused in several molecular approaches, some of them studying the developmentally regulated *Echinococcus* genes, as those concerning the stage-specifically regulated *14-3-3* gene in *E. multilocularis* (Siles-Lucas *et al.* 1998). *14-3-3* genes have been related with aberrant cellular activity, as the corresponding proteins regulate the activity of other molecules involved in abnormal cell proliferation (e.g. Pallas *et al.* 1994). Thus, we have suggested a possible relationship between 14-3-3 over-expression and the unlimited growth of the *E. multilocularis* metacestode (Siles-Lucas *et al.* 1998). Based on the above-mentioned differences in the growing behaviour between *E. granulosus* and *E. multilocularis* cysts, we decided to compare the expression level of the *14-3-3* gene in the metacestodes from those two species. Our results demonstrate that, compared to *E. granulosus* metacestode, the 14-3-3 protein is over-expressed in the metacestode of *E. multilocularis*. Furthermore, the corresponding native protein could not be detected in the germinal layer of *E. granulosus* cysts. In addition, the alignment of the *Echinococcus 14-3-3* protein sequence with different

14-3-3 proteins from other organisms allows including the parasite protein into the tumour growing-related 14-3-3 group. An actual co-operational role of the over-expressed 14-3-3 molecule in the tumour-like growth capacity of *E. multilocularis* metacestode is suggested.

#### MATERIALS AND METHODS

##### Parasites

*Echinococcus granulosus* metacestode tissue (cyst wall and protoscoleces) was collected from a fertile lung cyst (isolate CW1), obtained from a naturally infected cow from a local abattoir as described elsewhere (Felleisen & Gottstein, 1993).

##### In vitro culture

Microcysts of *E. granulosus* were obtained by *in vitro* cultivation of 50000 protoscoleces (isolate CW1, see above). The parasite was cultured basically as described before (Casado & Rodriguez-Caabeiro, 1989; Hemphill & Gottstein, 1995). Cysts were collected at day 90 of culture.

##### Parasite extracts, SDS-PAGE and immunoblotting

Somatic protein (S) extracts from *E. granulosus* protoscoleces and S extracts from *E. granulosus* cyst wall proteins, and total RNA from *in vitro*-obtained microcysts, were prepared as previously described (Siles-Lucas *et al.* 1998). S extracts from *E. multilocularis* protoscoleces and S extracts and RNA of cyst wall from *in vitro* recovered *E. multilocularis* microcysts (isolate Faub), obtained as described elsewhere (Casado & Rodriguez-Caabeiro, 1989; Hemphill & Gottstein, 1995), were a kind gift from Drs B. Gottstein, R. Felleisen and A. Hemphill (Institute of Parasitology, University of Berne, Switzerland). Methods for the extraction of proteins and RNA were performed as described elsewhere (Siles-Lucas *et al.* 1998). Following determination of protein concentration (Bradford, 1976), equal amounts of the above-mentioned protein extracts were separated in 12% SDS-PAGE under reducing conditions. After transfer of proteins onto nitrocellulose membranes (Gibco), immunoblot analyses were performed as described elsewhere (Siles-Lucas *et al.* 1998).

##### Reverse transcription (RT), polymerase chain reaction (PCR) and sequence analysis

A PCR-based approach was employed for amplification of the cDNA encoding the *E. granulosus* 14-3-3 protein. For amplification of the sequence between bases 58 and 909, total RNA from *in vitro*-obtained *E. granulosus* microcysts was reversely transcribed

into single-stranded cDNA as described elsewhere (Felleisen & Gottstein, 1994). The resulting cDNA was amplified as previously described (Siles-Lucas *et al.* 1998) using the gene-specific primers 1433-1 (5'-GC(C/T)AA(A/G)CTTGC(C/T)GA(A/G)CA-(A/G)GC) and 1433-2 (5'-CTCAATCAGAACCA-CGACAG), or the primer 1433-3 (5'-GAGAAA-ATTGGGTGCTGAAGC) in combination with an oligo-dT primer (dT16mer, Pharmacia). The sequence of the 5'-end of the cDNA encoding *E. granulosus* 14-3-3 (bases 1-365) was determined by sequencing of the PCR product obtained by amplification of 1  $\mu$ l ( $\sim 10^7$  plaque-forming units) of a  $\lambda$ gt11 *E. granulosus* protoscoleces cDNA library as template with the  $\lambda$ gt11 forward (Gibco) and the 1433-4 (5'-GCTTCAGCACCAATTTTCTC) primers. The 1433 primers were designed based on of the known *E. multilocularis* 14-3-3 cDNA sequence (accession no. U63643). PCR products were sequenced with the Sequenase sequencing kit (Pharmacia) with respective primers. The same procedure was followed for confirmation of the *E. multilocularis* 14-3-3 cDNA sequence. Both amino acid sequences were compared between them and with the data-bank available protein sequences from the  $\zeta$  (accession nos D83037, D87660, AW517476, AF033312, AA858662 and P29361),  $\beta$  (accession nos AF043736, P29358, AW518256, D17446 and AAC14343) and  $\sigma$  (accession nos AAF36093, AAC52030 and P31947) 14-3-3 isoforms. Grouping of the *Echinococcus* 14-3-3 protein in the most related cluster was achieved by alignment of the active centres of the different 14-3-3 proteins (described by Siles-Lucas *et al.* 1998) using the CLUSTAL™ computer package, and constructing the corresponding dendrogram with the Treeview™ computer program.

For the amplification of a partial exon from the *EgactII* *E. granulosus* actin gene (Da Silva *et al.* 1993), the same cDNAs were amplified as previously described (Siles-Lucas *et al.* 1998) with the Act1 (5'-AC(C/T)AATTG(A/G)GATGA(C/T)ATG) and Act2 (5'-AAGCG(T/A)TC(G/C)TTGCC(A/G)A-TG) primers, derived from the *EgactII* cDNA sequence (accession no. L07774). The corresponding PCR products—Egac and Emac—were cloned into the pMOS (Amersham) vector. The resulting sub-cloned products were used for transformation of *Escherichia coli* XL1B competent cells. After selection of recombinants and extraction of plasmid DNA, inserts were sequenced with the Sequenase sequencing kit (Pharmacia), with the T7 primer (Gibco).

##### Semi-quantitative RT-PCR and Northern blot

Total RNA from *in vitro*-cultivated *E. granulosus* and *E. multilocularis* microcysts was used for the comparison of the 14-3-3 gene expression levels between both metacestode tissues (germinal layer).

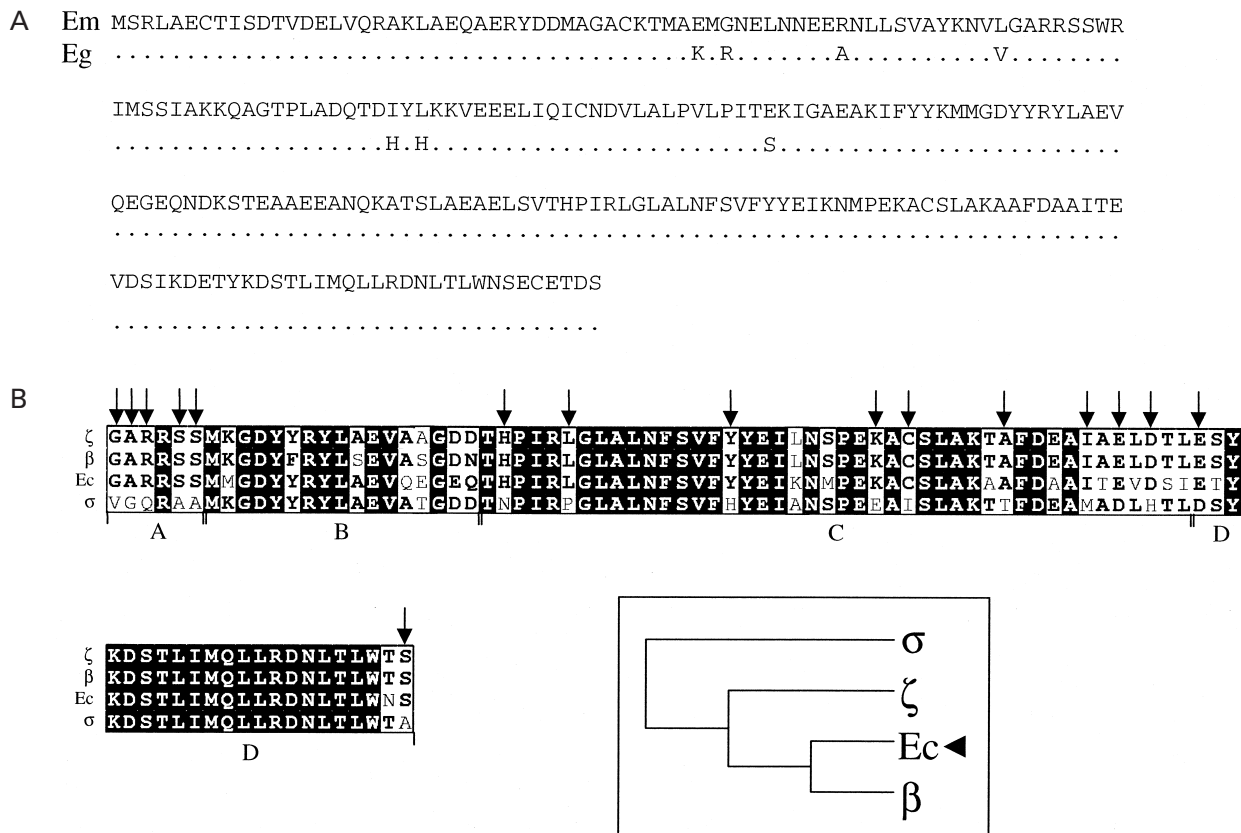


Fig. 1. (A) Aligned amino acid sequences of the *Echinococcus multilocularis* (Em, accession number U63643) and *E. granulosus* (Eg) 14-3-3 proteins. Dots indicate identity between both sequences. (B) The consensus sequence (Ec) from the active centres (A, protein kinase C pseudosubstrate; B, carboxylic terminus of annexins; C, interaction site with Ras; D, 14-3-3 feature, as described by Siles-Lucas *et al.* 1998) of both parasite sequences where aligned and genetically compared with the corresponding consensus sequences of group 1 ( $\zeta$  and  $\beta$  14-3-3 isoforms) and 2 ( $\sigma$  14-3-3 isoforms; see accession numbers in the Materials and Methods section). Homologous positions in the *Echinococcus*,  $\zeta$  and  $\beta$  isoforms, but different from  $\sigma$  isoforms, are marked with arrows. Homologous positions in all sequences are represented in white, black-background letters. The arrowhead in the corresponding dendrogram shows the genetic position of the *Echinococcus* 14-3-3 sequence, clustered into group 1.

After measurement of their concentration by spectrophotometry, RNAs were reversely transcribed into single-stranded cDNA as previously described (Felleisen & Gottstein, 1994). Following estimation of the cDNA concentration in each sample, the cDNAs were amplified as described before (Siles-Lucas *et al.* 1998) with the 1433.2 (above-mentioned) and 1433.5 (5'-ACATTTACCTTAAGAAGGTG) primers, annealing at fully homologous positions in the *E. granulosus* and *E. multilocularis* 14-3-3 cDNAs. The corresponding internal control (from the constitutively expressed *actinII* gene) products were obtained by amplification of the above-mentioned cDNAs, as described by Siles-Lucas *et al.* (1998), with the fully *E. granulosus* and *E. multilocularis* homologous Act3 (5'-ACCTTCTACAACGAAGTACG) and Act4 (5'-ATCTGGCAGTTCATAAGAGC) primers, designed after sequencing of the Egac and Emac PCR products (see above). cDNA serial dilutions were used in parallel as templates for both PCR reactions in order to determine the respective cut-off points. The amount

of 14-3-3 transcripts in the different samples could thus be compared in relation to the respective actin-specific cut-off point. The same total RNAs were used for Northern blot analysis as previously described (Siles-Lucas *et al.* 1998). The specific DNA probes for the *Echinococcus* 14-3-3 and *EgactII* genes were prepared by random priming of the corresponding affinity purified RT-PCR-specific products described above. Comparative quantitation of 14-3-3 and *EgactII* PCR-specific products or specific mRNAs from *E. granulosus* and *E. multilocularis* microcysts was done by densitometry, with the Kodak-Image<sup>™</sup> computer program.

#### Production of recombinant proteins

The Eg14t and Em14t peptides, corresponding to the N-terminally truncated *E. granulosus* and *E. multilocularis* 14-3-3 proteins, respectively, were obtained as described before (Siles-Lucas *et al.* 2000). After purity checking by SDS-PAGE, recombinant proteins were used for immunization of

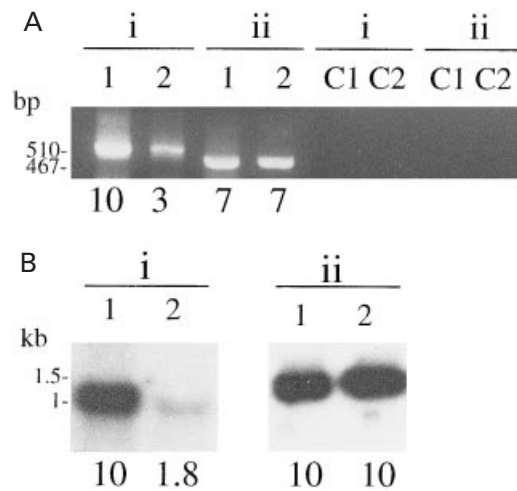


Fig. 2. (A) Semi-quantitative RT-PCR (i) of the *14-3-3* and (ii) of the constitutively expressed *EgactII* genes of transcripts derived from RNA of (1) *Echinococcus multilocularis* and (2) *E. granulosus* *in vitro*-obtained microcysts. C1 and C2 are negative controls of the amplification reaction with non-reversely-transcribed RNAs and without DNA template, respectively. Fragment sizes are indicated in base pairs (bp). (2b) Northern hybridization of total (1) *E. multilocularis* and (2) *E. granulosus* RNAs probed with (i) *14-3-3* and (ii) actin gene-specific fragments (see Materials and Methods section). Transcript sizes are indicated in kilobases (kb). Intensity of bands, calculated by densitometry, are indicated as numbers at the bottom of each band.

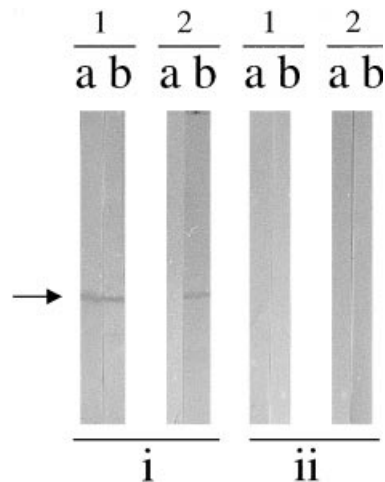


Fig. 3. Detection by immunoblot of the native *14-3-3* protein (marked with an arrow) in (a) cyst wall and (b) protoscoleces from (1) *Echinococcus multilocularis* and (2) *E. granulosus*, after SDS-PAGE in 12% gels under reducing conditions. (i) Reactivity of the anti-Eg14t hyperimmune mouse serum and (ii) control reactivity of the corresponding pre-immune mouse serum against respective protein extracts. Equal amount of proteins was loaded for all samples.

laboratory animals, as described elsewhere (Siles-Lucas *et al.* 2000). Sera were used for detection of the native *14-3-3* protein in different parasite tissues.

### Immunohistochemistry

Immunolocalization of the native *14-3-3* protein was attempted in cyst wall and protoscoleces from *E. granulosus* collected from a fertile cow lung cyst (isolate CW1, see above) in fixed, paraffin-embedded parasitic material. This material was serially sectioned at 10  $\mu$ m, and treated sections (as described by Thompson, Dunsmore & Hayton, 1979) were used for the immunological reaction with the anti-Eg14t mice sera, basically as described elsewhere (Rodrigues *et al.* 1997). Sections were also stained with haematoxylin-eosin, as described previously (Thompson *et al.* 1979).

### RESULTS

#### Sequence analysis of the *E. granulosus* and *E. multilocularis 14-3-3 cDNAs*

Sequencing of the *E. granulosus 14-3-3* cDNA revealed a continuous open reading frame of 244 amino acids (Fig. 1), in which the characteristic *14-3-3* family motifs were detected. The *E. multilocularis 14-3-3* cDNA sequence (accession number U63643) was confirmed and updated. The overall identity between the *E. granulosus* and *E. multilocularis* amino acid sequences was 97.5%. Comparative analysis of the *Echinococcus 14-3-3* sequence and different *14-3-3* isoforms showed a higher similarity of the parasite sequence with the  $\zeta$  and  $\beta$  isoforms, and a lower similarity with the  $\sigma$  isoforms (Fig. 1).

#### RT-PCR and Northern blot comparative analyses

RT-PCR amplification of the *EgactII* gene produced the expected 467 bp band in both *E. granulosus* and *E. multilocularis* metacestode reactions, with comparable intensity in both samples (Fig. 2). The *14-3-3*-specific amplification of the same samples gave the expected 510 bp bands, being the intensity of the PCR product remarkably higher for the *E. multilocularis* cDNA sample. No amplification was detected when non-reversely transcribed RNA or when no template was added to the PCR reaction (Fig. 2). RT and PCR reactions were repeated at least 3 times. The corresponding PCR specific fragments from the *14-3-3* and actin genes were used as probes for Northern hybridization of the same RNAs used for RT-PCR. Both probes gave single labelled bands of approximately 1 (*14-3-3*) and 1.5 (actin) kb for both *E. granulosus* and *E. multilocularis* RNAs. The intensity of the actin-specific bands was comparable for both samples, while the *14-3-3* band was notably more intense for *E. multilocularis* than for *E. granulosus* metacestode RNAs (Fig. 2). On the basis of the RNA and cDNA concentrations used for RT-PCR, the RT-PCR reaction cut-off points, and the densitometry analysis of the corresponding PCR

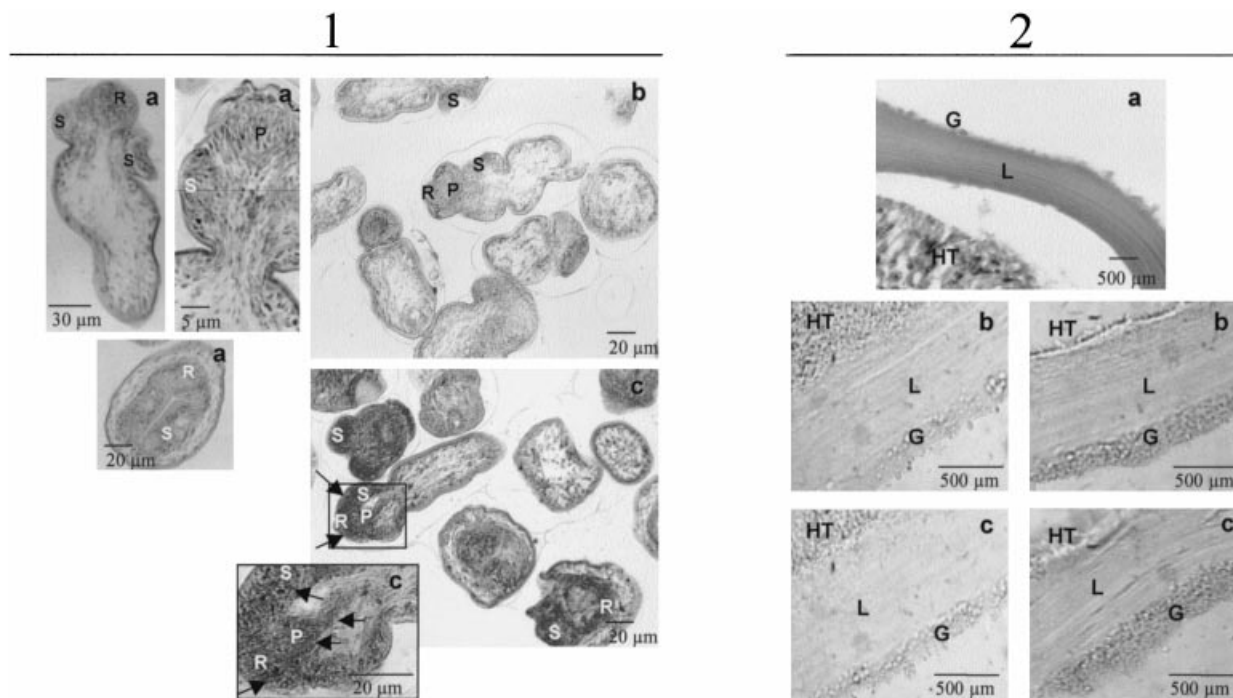


Fig. 4. Immunolocalization of the native 14-3-3 protein in *Echinococcus granulosus* (1) protoscoleces and (2) cyst wall. Sections were stained with (a) haematoxylin-eosin or probed with (b) a pre-immune or (c) the anti-Eg14t mouse serum. Anti-14-3-3 reactivity, marked with arrows, was detected in the pad and adjacent structures, and in the suckers (1c, see squared magnification in the bottom). No anti-14-3-3 reactivity was detected in germinative or laminated cyst layers (2c, two different sections). R, rostellum; P, pad; S, sucker; L, laminated layer; G, germinative layer; HT, host tissue.

bands and the transcripts detected by Northern blot, the expression level of the 14-3-3 gene in the metacestode of *E. multilocularis* can be estimated to be about 4 times higher than in the metacestode of *E. granulosus*.

#### Immunoblotting

Detection of the native *Echinococcus* 14-3-3 protein was achieved by immunoblot analysis with several hyperimmune anti-*Echinococcus* 14-3-3 mice sera. Under reducing conditions the native 14-3-3 protein, with an apparent molecular mass of about 24.5 kDa, was detected on blots containing *E. granulosus* and *E. multilocularis* protoscoleces extracts, and on *E. multilocularis* cyst wall extracts (Fig. 3). The protein was not detected in *E. granulosus* cyst wall extracts (Fig. 3).

#### Immunohistochemistry

The detection of the 14-3-3 protein in sections of *E. granulosus* protoscoleces with the anti-Eg14t mouse serum 14a resulted in a strong reactivity localized in areas detected with the haematoxylin-eosin stain as those with the highest cellular density (Fig. 4). No positive reaction was observed in sections of *E. granulosus* cysts, neither in the laminated nor in the germinal layer, as shown in Fig. 4. Similar results were obtained with the 14b to 14e anti-Eg14t mice

sera (data not shown). No reactivity was detected when the corresponding pre-immune mice sera were used for the immunological reaction (Fig. 4).

#### DISCUSSION

Employing a PCR-based strategy, we sequenced the complete 14-3-3 *E. granulosus* cDNA. The comparative sequence analysis demonstrated its high homology (almost identity) with the corresponding gene in *E. multilocularis* (Siles-Lucas *et al.* 1998).

14-3-3 proteins have been repeatedly associated with tumour processes. This was frequently demonstrated by showing the interaction of 14-3-3 proteins with promoters of oncogenic events – e.g. the middle-tumour antigen (Pallas *et al.* 1994), the  $\epsilon$  subtype of the protein kinase C (PKC $\epsilon$ ; Acs *et al.* 1995), the cMyc (Tzivion, Luo & Avruch, 1998) and the Raf-1 (McPherson *et al.* 1999). The activity of the above-mentioned molecules depends on their interaction with 14-3-3 proteins and is enhanced when 14-3-3 proteins are over-expressed. Over-expression of 14-3-3 proteins has also been directly associated with lung tumours, being a monoclonal anti-14-3-3 antibody the election tool for the diagnosis of this disease in sputum samples (Nakanishi *et al.* 1997).

We have previously described that the 14-3-3 protein is over-expressed by the metacestode of *E. multilocularis* in comparison with the adult worm, and mainly localizes in the germinal layer of the cyst

(Siles-Lucas *et al.* 1998). As mentioned, the *E. multilocularis* metacystode growth is characterized by the unlimited infiltration of its germinal layer within the host tissue. A possible role of the over-expression of the 14-3-3 protein in mediating the uncontrolled *E. multilocularis* (germinal layer) metacystode growth was thus suggested. The rate of the *E. granulosus* metacystode growth differs markedly when compared to that of the *E. multilocularis* metacystode. In *E. granulosus*, it is slow and limited by the host-derived adventitial layer (Thompson & Lymbery, 1995). However, no experimental molecular-based difference related with these very distinct growth behaviours has been described so far. Thus, our aim was to gain an initial insight on the possible role of the level of 14-3-3 protein expression in promoting the uncontrolled growth of *E. multilocularis* cyst, when compared with the non-metastatic *E. granulosus* metacystode.

To avoid host influence in the parasite development (Thompson & Lymbery, 1995), we obtained parasite material using an *in vitro* cultivation model (Casado & Rodriguez-Caabeiro, 1989; Hemphill & Gottstein, 1995). By a semiquantitative RT-PCR assay and Northern blot on total RNAs isolated from these *in vitro* obtained microcysts, we were able to detect a 4-fold 14-3-3 over-expression in the metacystode of *E. multilocularis* compared to *E. granulosus*. By immunoblotting, the native 14-3-3 protein could not be detected in cyst wall extracts of *E. granulosus*. On the contrary, a strong reactive band was found in the *E. multilocularis* cyst wall extracts. A band corresponding to the same size was also detected in protoscoleces extracts from both species. When detection of native protein was attempted on parasite sections, no signal showed up in laminated or germinal *E. granulosus* cyst layers, while the protein could be detected in protoscoleces from this species. As it is known, a second *Echinococcus* 14-3-3 isoform is produced by adult worms (Siles-Lucas *et al.* 2000). Thus, it remains to be clarified whether protoscoleces share one of the already characterized *Echinococcus* 14-3-3 isoforms or whether they possess their own. As it was previously demonstrated, the 14-3-3 protein can be readily visualized in the tumour-like proliferative *E. multilocularis* germinal layer (Siles-Lucas *et al.* 1998). These divergences in 14-3-3 expression and localization could in fact be related with the referred growth rate differences between both metacystodes.

As mentioned, 14-3-3 proteins have been associated with tumour processes. The  $\sigma$  isoforms have been found to be down-regulated in several carcinoma types, or over-expressed after gamma irradiation of cells (e.g. Hermeking *et al.* 1997; Ostergaard *et al.* 1997). On the other hand,  $\beta$  and  $\zeta$  isoforms are remarkably over-expressed by tumour cells, and they have been related at molecular level with an extraordinary activation of the oncogenic

PKC $\epsilon$  protein when over-expressed (Acs *et al.* 1995; Setoguchi *et al.* 1995; Nakanishi *et al.* 1997). Other molecular relationships between over-expressed 14-3-3 proteins and different oncogenic molecules have been repeatedly demonstrated. Unfortunately, details about 14-3-3 isoforms involved in such interactions are mostly lacking. Trying to correlate further the 14-3-3 over-expression in *E. multilocularis* cysts and its tumour-growth behaviour, we aligned the active centres of the parasite protein sequence with the corresponding available amino acid sequences from the 'pro-tumourigenic' ( $\beta$  and  $\zeta$ , called here 'group 1') and 'anti-tumourigenic' ( $\sigma$ , called here 'group 2') 14-3-3 isoforms. In the corresponding dendrogram, the *Echinococcus* 14-3-3 sequence is assigned to the group 1 ('pro-tumourigenic' isoforms).

In conclusion, we demonstrated the over-expression of the 14-3-3 protein in the tumour-like growing *E. multilocularis* metacystode, when compared with the slow-rate growing *E. granulosus* cysts. The over-expressed protein mainly localizes in the germinal layer of the parasite, which is in fact the structure responsible for the tumourigenic growth of the metacystode. The respective protein was not detectable in the germinal layer of the *E. granulosus* cysts. In addition, the parasite 14-3-3 protein could be genetically grouped with the 'pro-tumourigenic' 14-3-3 isoforms. Thus, we suggest the involvement of the over-expressed 14-3-3 protein in the promotion and/or maintenance of the progressive growth capacity of the larvae of *E. multilocularis*.

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