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The 14-3-3 protein is secreted by the adult worm of *Echinococcus granulosus*

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SUMMARY

The 14-3-3 protein, already described in the metacestode of Echinococcus multilocularis*, has been characterized in the* Echinococcus granulosus *adult worm. Immunolocalization studies show the presence of the 14-3-3 protein in the periphery of testes and externally associated with the apical rostellum and adjacent worm tegument. The alcian blue staining in consecutive parasite sections gave similar reactivity patterns, suggesting that the 14-3-3 protein is produced and secreted by rostellar glands. Immunoblot analysis showed the presence of the 14-3-3 protein in somatic and excretory-secretory worm products with higher and smaller apparent molecular masses, respectively, than those detected in* E. multilocularis *or* E. granulosus *metacestode tissues. Conversely, the 14-3-3 protein was not detected in metacestode secretory products. Detection of anti-*E. granulosus *14-3-3 reactivity in sera of experimentally infected dogs was achieved at early stages of infection. Specific antibody titres decreased during the course of infection. The possible origin and functions of the 14-3-3 protein produced by the adult worm are discussed.*

Keywords Echinococcus granulosus, *adult worm*, *14-3-3 protein*, *isoform*, *secretion*, *recognition by host sera*

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INTRODUCTION

The 14-3-3 proteins are a family of eukaryotic molecules, represented by different isoforms, which have been found in many organisms including parasites (e.g. Siles-Lucas *et al*. 1998). These proteins interact with several key-signalling molecules to regulate intracellular signal transduction events. The amino acid sequence of 14-3-3 proteins presents several conserved motifs potentially related with their activities (Aitken *et al*. 1995), among them the region which resembles the *C*-terminus of a group of chaperones called annexins. This region is potentially related with the regulation of the activities of Raf (Tzivion *et al.* 1998) and Cdc25 (Zeng *et al*. 1998) by 14-3-3 proteins, presumably acting as a molecular scaffold or chaperone. Other annexin-mimicking 14-3-3 activities, such as those related with secretion processes, have been described in adrenal chromaffin cells. Priming of catecholamine secretion in these cells by exogenous 14-3-3 treatment is apparently due to reorganization of the cell cortical actin network (Roth & Burgoyne 1995). Nevertheless, no secreted 14-3-3 protein isoform has been described to date.

Echinococcus granulosus is a small endoparasitic flatworm with an indirect life cycle. The adult worm develops in the small intestine of the definitive (canine) host deeply invading the crypts of Lieberkühn, and the larvae develop in the viscera of the intermediate host, represented by a wide range of mammals, including man, producing the unilocular hydatid cysts (Thompson 1995).

Because of the clinical and economical significance of this parasitic disease, major efforts have been made to extend knowledge of the biology of parasitism and host–parasite interactions in order to improve diagnosis, prevention and treatment of hydatid disease. Remarkably, these studies have been confined to infection with the larvae. Thus, little is known about host–parasite interactions or parasite metabolic activities at a molecular level regarding *Echinococcus* adult worms.

When established, the *Echinococcus* adult begins its secretory activity through a modified group of tegumental

cells, called rostellar glands, situated in the apical rostellum in a process similar to the holocrine secretion. The nature of this secretion remains unclear. This process has a particular significance in the host–parasite relationship, as it is possibly related to attachment, nutrition and protection of the parasite from the host immune effector mechanisms (Thompson 1995). Therefore, release of secretory material into the host–parasite interface is a basic event for the establishment and survival of *Echinococcus* worms.

The aim of the present work was to characterize the 14-3-3 protein in the adult stage of *E. granulosus*, describing its possible role in secretion processes and proposing the potential mechanism of this molecule in the secretory activity of the adult parasite.

MATERIALS AND METHODS

Parasites

Echinococcus granulosus metacestode tissue (cyst wall and protoscoleces) and hydatid fluid were collected as already described (Siles-Lucas *et al.* 1996) at a local abattoir, from a fertile lung cyst of a naturally infected cow (isolate CW1), characterized as sheep strain by random amplified polymorphic DNA technique (Siles-Lucas *et al.* 1994).

Parasite extracts

Extracts of somatic proteins (SP) from *E. granulosus* metacestode tissue (protoscoleces and cyst wall, isolate CW1) were prepared as described recently (Siles-Lucas *et al.* 1998). Extracts of SP from *E. granulosus* adult worms were a kind gift of Dr C. Chalar (Lab. Bioquímica, Fac. Ciencias, Montevideo, Uruguay). Excretory–secretory proteins (ESP) from *in vitro* cultured *E. granulosus* microcysts (Casado & Rodriguez-Caabeiro 1989) were prepared by concentration of respective culture supernatants on a Speed Vac (Pharmacia, Uppsala, Sweden). ESP from 48 h-*in vitro* cultured *E. granulosus* protoscoleces and 35 day-old adult worms (sheep strain isolate), obtained as described earlier (Malgor *et al.* 1997), were a kind gift of Drs H. Carol and S. González (Cátedra de Inmunología, Fac. Química, Montevideo, Uruguay). Extracts of cyst wall SP from *in vitro* obtained *E. multilocularis* microcysts (Hemphill & Gottstein 1995) were a kind gift from Dr A. Hemphill (Inst. Parasitology, University of Berne, Switzerland). Following determination of protein concentration by the Bradford (Bradford 1976) method, these extracts were used for immunoblot analyses.

Production of an *E. granulosus* recombinant 14-3-3 protein for immunization of laboratory animals

A polymerase chain reaction (PCR) was performed with reversely transcribed total RNA from *in vitro* cultivated *E. granulosus* microcysts, with specific primers 1433·2a (5′-CCCGCGGCCGCCTCAATCAGAACCACGACAG) and 1433·3a (5′-CCCGGGTCGACTGAGAAAATTGGTGCT GAAG) containing *Not*I and *Sal*I adapters, respectively, as described previously (Siles-Lucas *et al.* 1998). The corresponding 452 bp in length specific product and the pGEX (Pharmacia) vector were double-digested with *Not*I and *Sal*I restriction enzymes (Gibco BRL, Gaithersburg, USA) and affinity purified from preparative 1% agarose gels. These double-digested products were used for ligation reactions, performed with the linearized pGEX vector plus the specific PCR product as described previously (Sambrook *et al.* 1989) and the resulting ligation products were used for transformation of *E. coli* AD202 competent cells. After selection of recombinants and extraction of plasmidial DNA, inserts were sequenced with the Sequenase sequencing kit (Pharmacia). The expression of the corresponding GST-fused protein in AD202 cells was achieved as described elsewhere (Sambrook *et al.* 1989). The resulting *E. coli* extracts were incubated with the Glutathione-Sepharose affinity resin (Pharmacia) as described by the commercial supplier. After extensive washing of the resin with cold PBS and checking for the presence of bounded fusion protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970), thrombin (Pharmacia) cleavage of the GST-fusion protein was done as recommended by the commercial supplier. The resulting supernatant containing the corresponding recombinant protein, called Eg14t, was collected and protein content determined (Bradford 1976). After assesment of purity by SDS-PAGE, Eg14t was used for immunization of laboratory animals.

Antisera

The Eg14t recombinant protein was used to obtain five (14a to 14e) polyclonal sera. These were raised in mice by intraperitoneal injection of 40 μg protein solubilized in 50 μl PBS plus 50 μl Freund's complete adjuvant (Life Technologies, Bethesda, MD, USA). One boost at day 21 was performed with 40 μg of protein solubilized in 50 μl PBS plus 50 μl Freund's incomplete adjuvant (Life Technologies). Mice were bled at days 0 (preimmune serum) and 28 (hyperimmune serum). The sera were used for detection of native 14-3-3 protein in different parasite extracts and immunolocalization in *E. granulosus* adult worms.

Sera

Sera from four different dogs orally infected with 60 000 *E. granulosus* protoscoleces (as described elsewhere, Spinelli *et al.* 1996) were collected at 0, 5, 12, 15, 23, 26 and 29 days postinfection (d.p.i.) and analysed for the presence of anti-Eg14t antibodies by ELISA and immunoblot.

Immunohistochemistry

Native 14-3-3 protein was immunolocalized in 35 day-old adult worms in a fixed, paraffin embedded small intestine fragment from a dog experimentally infected with *E. granulosus*, prepared as already described (Thompson *et al.* 1979). The paraffin-embedded material was serially sectioned at 10 μm, and treated (Thompson *et al*. 1979) sections were used for the immunological reaction with the anti-Eg14t recombinant protein mice sera (Rodrigues *et al*. 1997). Several sections were also stained with haematoxylin-eosin or, after a performic acid treatment, with alcian blue (Thompson *et al*. 1979).

SDS-PAGE and immunoblotting

E. granulosus hydatid fluid proteins, SP metacestode tissue (isolate CW1) and 35-day-old adult worm extracts, ESP from *in vitro* obtained/maintained *E. granulosus* microcysts, protoscoleces or adult worms and SP cyst wall from *E. multilocularis in vitro* obtained microcysts, as well as recombinant Eg14t protein, were separated in 12% SDS-PAGE (Laemmli 1970) gels under reducing conditions. For immunoblot analyses, transfer of proteins to nitrocellulose membranes (Gibco) was achieved by the Western-blotting technique (Towbin *et al.* 1979). For immunological reactions, anti-Eg14t mice or *E. granulosus* experimentally infected dog sera were used at 1 : 1000 and 1 : 100 dilution, respectively. Immunoreactive bands were visualized with peroxidase-labelled antimouse or antidog conjugate (Dako) at 1 : 1000 dilution. Immunoblots were developed by standard procedures (Harlow & Lane 1988). Duplicate gels were stained with Coomassie-blue.

ELISA

ELISAs were performed as previously described (Siles-Lucas *et al*. 1998). Briefly, ELISA-plates (Nunc, Roskilde, Denmark) were coated with 500 ng Eg14t per well. The above-mentioned dog sera were tested at 1 : 100 dilution. Peroxidase-labelled antidog conjugate (Dako, Glostrup, Denmark) was used at 1 : 1000 dilution. Cut-off points for each dog were calculated and the medium of five independent OD values from respective preimmune sera + 2 SD.

RESULTS

Immunohistochemistry

Detection of 14-3-3 protein in sections of *E. granulosus* adult worms with the anti-Eg14t mouse serum 14a resulted in a rather weak reactivity localized in the external layer of testis from mature proglottids, marked with arrows in Figure 1.1(C). A stronger reactivity was detected associated with the apical area (rostellum) of the worm and at the outer, adjacent portion of the tegument (Figure 1.2.1C). After performic acid treatment of sections, the reaction, marked in Figure 1.2.2(C), was still clearly detectable in the apical rostellum, either at the host–parasite interface or still inside the parasite tissue. This last reactivity was similar to that obtained by Alcian Blue staining of consecutive sections, marked with white arrows in Figure 1.2.2(D). Similar results were obtained with the 14b to 14e anti-Eg14t mice sera (data not shown). No reactivity was detected when the corresponding preimmune sera were used for the immunological reaction (Figure 1.1.1B, 1.2.1B and 1.2.2B).

Detection of the native *E. granulosus* 14-3-3 protein in different parasite extracts by immunoblot

For the detection of native *Echinococcus* 14-3-3 protein, an immunoblot analysis was carried out with several hyperimmune mice sera raised against the Eg14t. Using serum 14a (Figure 2b), the native protein was detected on blots containing SP from *E. granulosus* metacestode tissue and *in vitro* obtained *E. multilocularis* microcysts, and on SP and ESP from *E. granulosus* adult worms. The apparent molecular mass of the native protein was slightly higher, with a weak reactivity, in SP from *E. granulosus* adult worms, and smaller, with a stronger reactivity, in ESP from *E. granulosus* adult worms, when compared with the molecular mass of the protein detected in SP from *E. granulosus* or *E. multilocularis* metacestode tissue extracts, which conversely showed the same reactivity and apparent molecular mass of 24·5 kDa. On the other hand, the 14-3-3 protein was not detected in *E. granulosus* hydatid fluids, microcyst or protoscoleces culture supernatants (Figure 2b). Sera 14b to 14e gave similar results (data not shown). These hyperimmune mice sera specifically recognized the recombinant Eg14t protein (Figure 3b,c).

ELISA

An ELISA test was performed for the detection of anti-Eg14t antibodies in sera of *E. granulosus* experimentally infected dogs. All tested dogs recognized the Eg14t protein precociously at day 5 p.i. The reactivity of sera decreased or

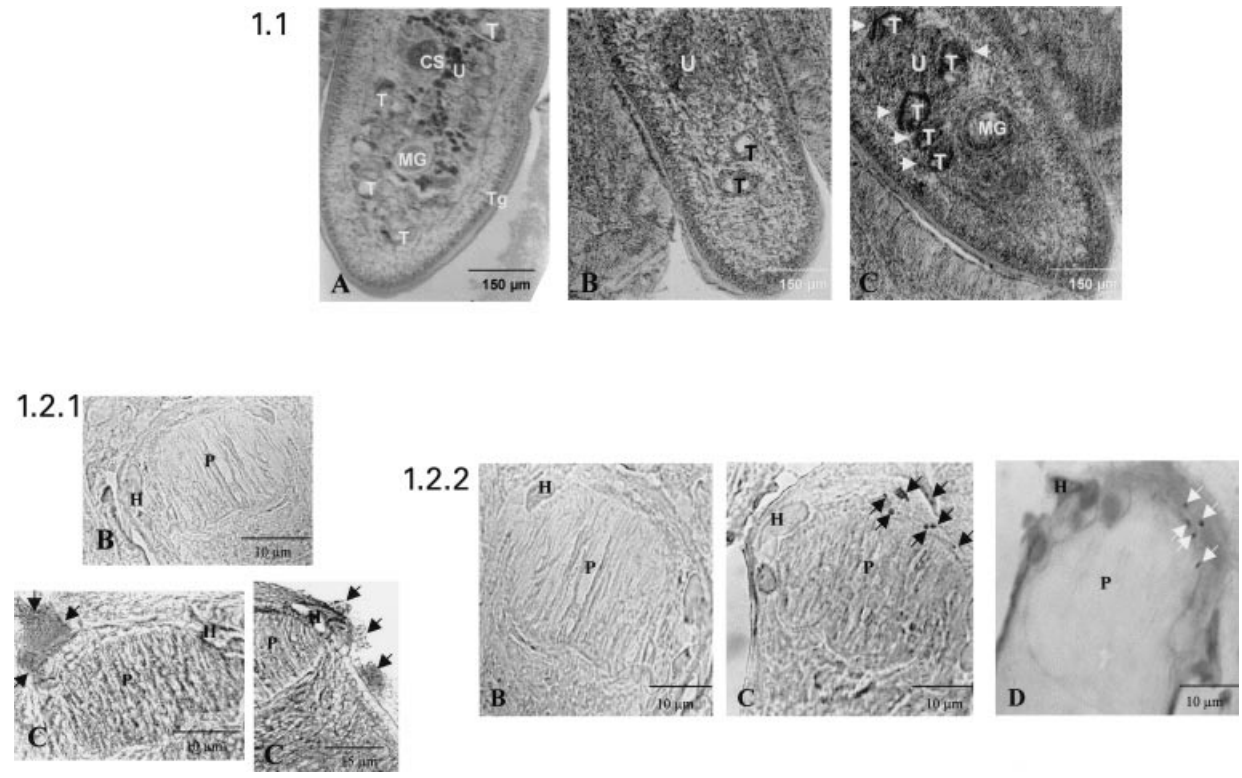

Figure 1 Immunolocalization of the 14-3-3 protein in 35 day-old *Echinococcus granulosus* adult worms longitudinal sections. Sections were stained with haematoxylin-eosin (A), probed with a preimmune (B) or the polyclonal anti-Eg14t (C) mouse serum. Anti-14-3-3 reactivity, marked with arrows in C, was detected in the periphery of testis inside the mature proglottid (1.1C) and externally associated with the apical rostellum and adjacent tegument (1.2.1C). In (1.2.2), sections were previously treated with performic acid and subsequently probed with the above-mentioned sera (B,C) or stained with alcian blue (D), which reactivity, marked with arrows, is identical to that obtained with the anti-Eg14t mouse serum (C), also marked with arrows.T, testes; U, uterus; Tg, tegument; CS, cirrus sac; MG, Melhis gland; H, hook; P, rostellar pad; S, sucker.

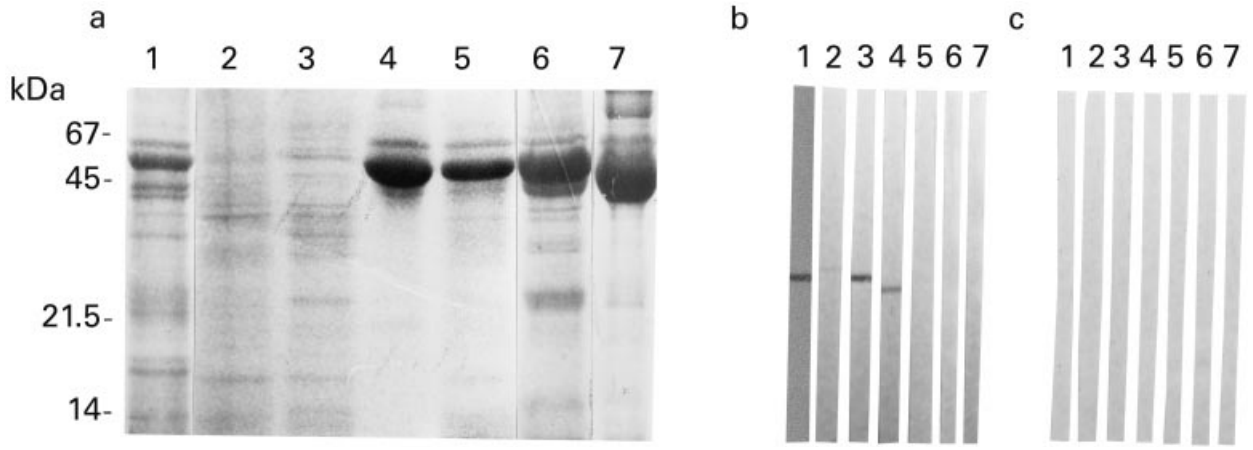

Figure 2 Detection of 14-3-3 native protein in *Echinococcus multilocularis* cyst wall (1), *E. granulosus* adult worm (2) and metacestode tissue somatic extracts (3), and in *E. granulosus* secretory compounds from adult worm (4) and protoscoleces (5), hydatid fluid (6) and microcysts culture supernatant (7). (a) Coomassie blue-stained proteins after SDS-PAGE in 12% gels under reducing conditions; (b) reactivity of the anti-Eg14t mouse serum against respective protein extracts, by immunoblot; (c) control reactivity of the preimmune mouse serum against respective protein extracts, by immunoblot. Molecular masses of the proteins are indicated in kDa.

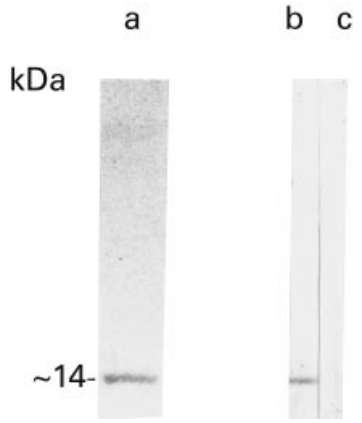

Figure 3 SDS-PAGE of the Eg14t protein, Coomassie blue stained (a) and after immunoblot with the anti-Eg14t hyperimmune (14a to 14e pool) mice sera (b) and respective pool of preimmune mice sera (c).Molecular mass of the recombinant protein is indicated in kilodaltons (kDa).

became negative in successive d.p.i. (Figure 4a). A pool of ELISA-positive sera showed reactivity by immunoblot against the Eg14t protein (Figure 4b,c).

DISCUSSION

The 14-3-3 gene has already been described and partially characterized in the metacestode of the related species *E. multilocularis*. The corresponding protein, detected in metacestode tissues, is overexpressed by the germinative layer of the *E. multilocularis* metacestode, when compared with the adult worm of the same species (Siles-Lucas *et al*. 1998). In this work, the 24·5 kDa native 14-3-3 protein could not be detected in somatic extracts of adult worms, using different antimetacestode hyperimmune rabbit sera. The 14-3-3 proteins are ubiquitous in eukaryotes and they are normally represented by several isoforms in each organism (Aitken *et al*. 1992). Thus, undetectability of the protein in *Echinococcus* adult worms could be attributed not only to a lower expression compared to metacestodes, but also to the presence of other, adult-specific 14-3-3 isoforms, with a different relative molecular mass. To assess this hypothesis, we performed the preliminary characterization of the *E. granulosus* adult worm specific 14-3-3 protein, by immunolocalization and immunoblot.

By immunohistochemistry, we were able to detect the 14-3-3 protein associated with the outer apical area (rostellum) and neighbouring tegumental surface of the adult worm. The appearance and localization of this reactivity strongly resembled those already detected by histochemistry in rostellar *E. granulosus* adult worm ES compounds (Thompson *et al*. 1979) and apical-associated ES products from other cestodes (Featherson 1972, Andersen 1975).

The rostellar gland parasite secretion plays an important role in the host–parasite interactions in the *Echinococcus* infection of the definitive host. Possible functional activities of this secretion could be associated with nutrition, adhesion

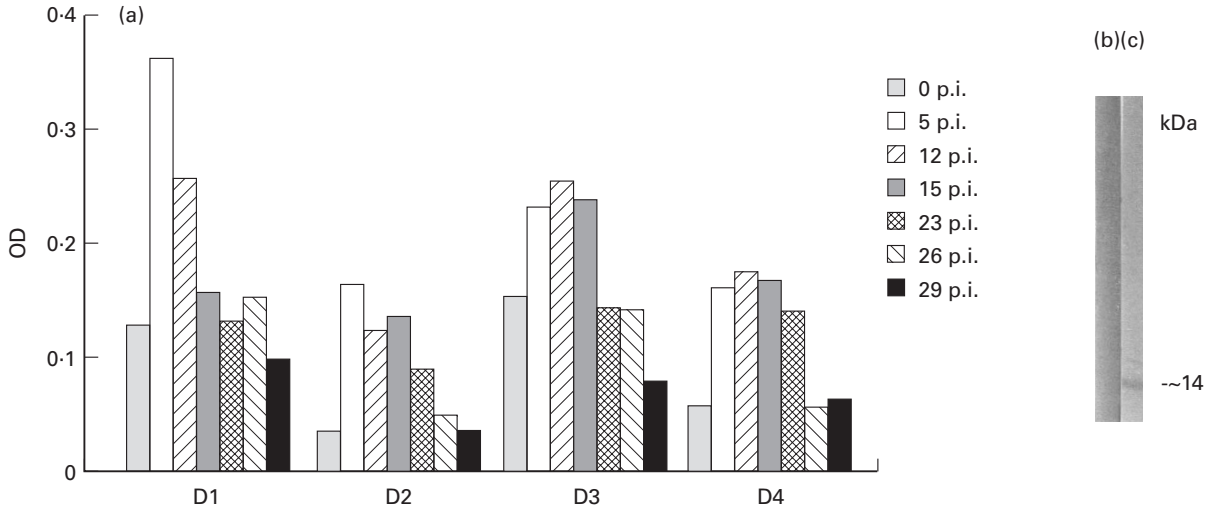

Figure 4 (a) ELISA reaction of the Eg14t recombinant protein with sera of dogs experimentally infected with *E. granulosus*, bled at different days postinfection, indicated on the right of the figure. D, dog; p.i., postinfection. Negative (cut-off) bar (0 p.i.) for each dog represent quintuplicate values of preimmune sera plus two standard deviations. (b) Reactivity of a pool of preimmune (D1 to D4, 0 p.i.) and (c) immune (D1 to D4, 12 p.i.) sera of dogs experimentally infected with *E. granulosus* against the Eg14t protein, by immunoblot. Molecular mass of the recombinant protein is indicated in kDa.

and protection of the *Echinococcus* worm from host immune responses (Thompson 1995). Although the nature of the rostellar gland secretion remains unclear, it is known to be constituted of cysteine-rich proteins and thus susceptible to staining with Alcian Blue (Thompson *et al*. 1979). The Alcian Blue staining technique requires the previous treatment of sections with performic acid. After such treatment, it was still possible to detect anti14-3-3 reactivity at the apical region of the worm. This reactivity was very similar to that in serial tissue sections stained with the Alcian Blue dye. Thus, we assume that the 14-3-3 protein is produced in the rostellar glands and secreted by the *E. granulosus* adult worm, and so released into the host–parasite interface. In this case, the 14-3-3 protein would then represent the first rostellar gland secretory compound characterized to date.

The 14-3-3 protein was also detected at the outer part of the structures called testes, situated into the mature proglottid of the *Echinococcus* worm. Because of the known high secretory activity of specific cells of this kind of organ in other organisms (Clark *et al*. 1991), we could infer that the 14-3-3 protein is also produced and secreted by the peripheral testis cells of the *E. granulosus* worm.

By an immunoblot analysis, we detected the native 14-3-3 protein both in the *E. granulosus* adult worm SP and ESP, with apparent molecular masses slightly higher and smaller, respectively, than the 24·5 kDa 14-3-3 native protein detected in *E. multilocularis* or *E. granulosus* metacestode SP. These results agree with those previously obtained, regarding detection of the native protein in *E. multilocularis* metacestodes with an apparent molecular mass of 24·5 kDa (Siles-Lucas *et al*. 1998). On the other hand, the protein was undetectable in *E. granulosus* hydatid fluids, in microcyst culture supernatants and in the supernatant of *in vitro* maintained protoscoleces. This strongly suggests that the 14-3-3 is not secreted (or secreted only at a very low level) by metacestode tissue-related structures.

As previously mentioned, 14-3-3 proteins can be represented by different isoforms in a single organism. The detection of the native protein in SP and ESP from the adult worm, with different apparent molecular masses compared to those detected in metacestode tissues, may suggest that the 14-3-3 protein produced by rostellar glands could indeed represent a new 14-3-3 adult worm-produced isoform, different from the one already characterized in the metacestode, which would potentially lose a signal peptide when secreted. The putative relationship of 14-3-3 proteins with the secretory-related molecules called annexins is reflected in the similarity of a highly conserved region of 14-3-3 with the C-terminus of those proteins (Aitken *et al*. 1995). This upholds the assumption of the potential cleavage of the *E. granulosus* adult-specific 14-3-3 protein, as *N*-terminal cleavage of several annexins has regulatory importance for the secretion process in other cell types (Mowitz *et al.* 1999).

It is well known that several types of annexins take part in exocytosis, contributing to the aggregation and fusion among secretion granules and between these and the plasma membrane, being released together with other ES compounds (Sarafian *et al*. 1991). Considering the *E. granulosus* adult 14-3-3 as an annexin-like molecule, we could therefore assume it to take part in the secretion in the apical worm tegument through such a mechanism.

The actual relationship between 14-3-3 proteins and secretory processes has also been described by several authors (Morgan & Burgoyne 1992, Wu *et al.* 1992, Roth *et al.* 1993, Chamberlain *et al*. 1995). It has also been demonstrated that the exogenous stimulation of chromaffin cells with 14-3-3 protein stimulates the catecholamine release of those cells (Roth & Burgoyne 1995). In this context, the 14-3-3 protein has already been detected in other fluids, such as sputum samples from lung cancer patients (Shoji *et al*. 1996) and cerebrospinal fluid of patients with prion-related and unrelated neurological diseases (Hsich *et al*. 1996, Beaudry *et al*. 1999, Satoh *et al*. 1999). Nevertheless, no secreted isoform of the 14-3-3 proteins has been described in other organisms so far. Thus, those authors attributed the presence of the 14-3-3 protein in exogenous fluids to the disruption of cells in the damaged tissues. This is consistent with the secretion mechanism proposed by other authors for the *Echinococcus* adult worm, which suggests the need of the disruption of the apical worm tegument to release the secretory compounds, followed by healing of this area (Thompson *et al*. 1979). In contrast, the present results suggesting the loss of a signal peptide in the process of 14-3-3 secretion, could argue for the potential existence of secreted 14-3-3 isoforms in *Echinococcus*, as well as in other organisms, in nondamaged healthy tissues. Thus, secretion would be an actively controlled process in the adult worm, without disruption of tissues, but rather by fusion of secretory granules with the cell membrane and subsequent release of molecules. This controlled process would also be in better accordance with the timing of the release of secretory compounds by the *Echinococcus* worm observed by other authors (Thompson *et al*. 1979). Concomitant damage and healing of the apical worm tegument cannot be ruled out, although migratory cells or molecules common to wound repair in other tissues should be demonstrated in the *Echinococcus* adult worm.

By ELISA tests, we detected anti-*Echinococcus* 14-3-3 antibodies in the sera of dogs experimentally infected with *E. granulosus*, at early stages of infection (5 d.p.i.), with a decrease of reactivity during the course of infection, which could be explained by the already described process of general and sequential depression of host immune responses

induced by the worm of *E. granulosus* (Heath 1995). Detection of early anti-*Echinococcus* antibodies (5 d.p.i.) in infected dogs has already been achieved by other authors, although decrease in antibody titres was not observed during the course of infection (Singh & Dhar 1988). Our results, indicating the progressive decrease of anti-Eg14t specific antibody titres during the infection course, may indicate a site-specific gradual depression of the immune response against some parasite molecules elicited at the most intimate host-parasite location or, more likely, a dramatic change in the quantity of some of the released molecules, including the 14-3-3.

It has been suggested that rostellar secretion activity coincided with a levelling off in the growth of the worm at around 30 d.p.i. (Thompson *et al*. 1979). The detection of anti-*E. granulosus* 14-3-3 protein antibodies in infected dogs already at day 5 p.i. suggests that the active secretion of rostellar compounds may initiate at early infection stages, assuming that this protein is part of the rostellar secretion. As it is known, protoscoleces are already situated within the crypts of Lieberkühn 3–6 h after infection (rev. in Thompson 1995). Thus, this early secretory activity could be related with a protective function of the secreted compounds, which would then operates throughout the life cycle of the adult worm, already beginning when an intimate relation between parasite and host take part. This is very likely, as maintenance of the integrity of worm structures which are in deep contact with the host tissue, as the rostellum, would suggest the production of local molecules capable of controlling or regulating the immune adverse responses of the host. Functions of this early secretion are also likely to be related with nutrition, as in the first days postinfection juvenile worms are metabolically very active (Thompson 1995). Therefore, uptake of suitable nutrients, digested by worm secretions, to refill reserves would be necessary for the survival of the parasite.

As known, the life cycle of *E. granulosus* involves two phases, the tissue-invading metacestode and the enteric adult, which have rather different immunological relationships with the host. Studies on the immune control of the metacestode infection by vaccination have recently been carried out (Lightowlers *et al*. 1999), but there are little prospects of a similar control of the infection with adult worms. The present results indicate the 14-3-3 *E. granulosus* adult-secreted protein to be a potential candidate for vaccination assays against the echinococcal disease in the definitive host, aspect that is already under study.

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