myo-Inositol hexakisphosphate is a major component of an extracellular structure in the parasitic cestode Echinococcus granulosus

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myo-Inositol hexakisphosphate (IP₆) is an abundant intracellular component of animal cells. In this study we describe the presence of extracellular IP₆ in the hydatid cyst wall (HCW) of the larval stage of the cestode parasite Echinococcus granulosus. The HCW comprises an inner cellular layer and an outer, acellular (laminated) layer up to 2 mm in thickness that protects the parasite from host immune cells. A compound, subsequently identified as IP₆, was detected in and purified from an HCW extract on the basis of its capacity to inhibit complement activation. The identification of the isolated compound was carried out by a combination of NMR, MS and TLC. The majority of IP₆ in the HCW was found in the acellular layer, with only a small fraction of the compound being extracted from cells. In the laminated layer, IP₆ was present in association with calcium, and accounted for up to 15% of the total dry mass of the HCW. IP₆ was not detected in any other structures or stages of the parasite. Our results imply that IP₆ is secreted by the larval stage of the parasite in a polarized fashion towards the interface with the host. This is the first report of the secretion of IP₆ and the possible implications beyond the biology of E. granulosus are discussed.

Key words: calcium, cestode, hydatidosis, inositol polyphosphates, phytic acid.

INTRODUCTION

Higher myo-inositol polyphosphates are ubiquitous components of animal cells. Of these, myo-inositol hexakisphosphate (IP₆) is the most abundant, with its concentration ranging from 10–100 μM in mammalian cells depending on cell type and developmental stage [1–4]. However, there are still major gaps in the understanding of this molecule. One of the important questions still to be answered is the complete elucidation of the pathways for IP₆ synthesis and degradation in animal cells. This has impaired the generation of knockout animals deficient in IP₆, thus making the physiological role of this molecule still a matter of debate [5,6]. The interaction of IP₆ with several intracellular proteins has been investigated by means of in vitro experiments [7–10]. In some cases, these interactions result in the inhibition [8,11] or potentiation [10] of the physiological activities of the proteins involved. The most compelling evidence arising from these studies suggests a role for IP₆ as a cofactor in DNA repair by non-homologous end-joining [10]. Recent studies in yeasts, using mutants deficient in IP₆ synthesis, have shown that the molecule is involved in mRNA export from the nucleus to the cytosol [12].

Another aspect of IP₆ biology that is not completely understood involves its cellular localization and the determination of whether the molecule is found in a free soluble form or in association with membranes or other cellular components. Cell permeabilization studies indicate that the majority of IP₆ in mammalian cells is present in, or in rapid exchange with, the cytosol, although the existence of a minor IP₆ pool in other membrane-bound compartments has not been excluded [13]. The unusual behaviour of IP₆ in solution [10,14,15] does not allow the prediction of whether the molecule can pass freely through nuclear pores. However, the suggestion that IP₆ has a role in the nucleus would imply that the molecule is present in this compartment. A further factor raising questions about the location of IP₆ arises from the finding that the only enzyme known to dephosphorylate IP₆, the multiple inositol polyphosphate phosphatase (MIPP), is present exclusively in the lumen of the endoplasmic reticulum (ER) [14]; the enzyme is nonetheless able to regulate the levels of cytosolic IP₆ to some extent [16]. These results suggest the existence of a low-affinity IP₆ translocator in the membranes of the ER. Finally, endogenously synthesized IP₆ has never been reported to be secreted from cells or to be present at high levels in extracellular compartments [5].

The current study demonstrates the presence of abundant extracellular IP₆ in the cyst wall of Echinococcus granulosus. This is a cestode parasite whose larval stage dwells within internal organs of livestock animals and humans. The larva has the form of a fluid-filled cyst (hydatid cyst) (Figure 1), which can reach up to 25 cm in diameter. The part of the cyst that is exposed to the host is the hydatid cyst wall (HCW). This structure is composed of two layers: the germinal and laminated layers. The innermost layer (germinal layer), which is only two or three cells thick, is the live parasite tissue and synthesizes the outer laminated layer. This layer is an acellular structure that resembles an exaggerated glycocalyx. It is a carbohydrate-rich meshwork that precludes host cells from direct contact with the parasite cells, but is permeable to host macromolecules. The germinal layer also gives...
rise, through budding towards the interior of the cyst, to the protoscoleces, which are the infective stage for the host in which the adult worm develops. Recently, during a study to identify molecular species from the hydatid cyst that interact with host immunity, we have isolated from the HCW a low-molecular-mass, heat-stable molecule with the capacity to inhibit complement activation [17]. In the present study, we describe the identification of this molecule as IP₆ and its localization to the laminated layer of the hydatid cyst.

MATERIALS AND METHODS

Materials

IP₆ was purchased from either Sigma (St Louis, MO, U.S.A.) or Calbiochem (La Jolla, CA, U.S.A.) and myo-inositol pentakis-phosphate (IP₅) was from Matreya Inc. (Pleasant Gap, PA, U.S.A.). Polyethyleneimine (PEI)-cellulose plates for TLC were obtained from Macherey–Nagel (Düren, Germany). The protease inhibitors PMSF, trans-epoxy succinyl-L-leucylamido(4-guanidino)butane (E-64), and benzamidine hydrochloride, as well as general chemicals, were purchased from Sigma.

E. granulosus extracts

‘Mother’ bovine cysts

Large cysts (several cm in diameter) were obtained from natural infections in cattle and are referred to as ‘mother’ bovine hydatid cysts. After puncture and aspiration of the cyst fluid, the HCW collapse, detaching from host tissue. HCW were generally used as the whole entity. In specified cases, the germinal and laminated layers were separated from each other with forceps. A soluble extract from whole bovine HCW was obtained by homogenization with a pestle and mortar under liquid N₂, followed by extraction for 2 h at 25 °C with PBS containing 5 mM EDTA, 1 mM PMSF, 1 mM benzamidine and 20 μM E-64 (PBS/EDTA). This extract was used for the initial purification of the component under study. In addition, isolated germinal and laminated layers were washed extensively with PBS and then extracted for 4 h at 25 °C with PBS/EDTA containing 0.5% (w/v) Tween 20 (PBS/EDTA/Tween) or PBS/EDTA respect-

vively. Extracts were clarified by centrifugation and kept for analysis.

Small cysts

We use the term small cysts to refer collectively to: (i) ‘daughter’ bovine cysts, which are hydatids that grow within larger cysts; and (ii) murine cysts, which were obtained from the peritoneal cavities of mice 8 months after intraperitoneal infection with protoscoleces [18]. Intact cysts (diameters ranging between 0.3–1.5 cm) were washed with PBS and then extracted with either PBS/EDTA for 3 h (‘daughter’ bovine cysts) or two separate PBS/EDTA washes for 1 h and 2 h respectively (murine cysts). Cyst fluid was removed by aspiration with a syringe, the HCW was opened with a scalpel blade and extracted at 4 °C with PBS/EDTA/Tween for 1 h (‘daughter’ bovine cysts) or for 30 min twice (murine cysts). Thus extracts were obtained by external washing of intact cysts and by detergent treatment of cysts opened to expose the cellular structures. Other murine cysts were extracted ‘from the inside’ by injecting EDTA solution so as to attain a final concentration equal to that used for extracting the cysts ‘from the outside’. After incubation at 25 °C for 3 h, the liquid inside the cyst was retrieved with a syringe.

Protoscoleces

Protoscoleces from bovine cysts were washed with PBS, homogenized under liquid N₂, and extracted with PBS/EDTA for 2 h at 25 °C. The residue was incubated under conditions (200 mM EDTA for two weeks at 4 °C) known to dissolve the ‘calcareous corpuscles’, a conspicuous feature of this stage of the parasite (M. Marín, unpublished work). The soluble extracts obtained after the two steps were kept for analysis.

Adult worms

Adult worms were recovered from intestines of dogs 32 days after oral infection with protoscoleces, as described previously [19]. The extract from these worms was a gift from Silvia Gonzalez (Cátedra de Immunología, Montevideo, Uruguay), and was obtained by extensive sonication in PBS containing 5 mM EDTA and 2 mM PMSF.

E. multilocularis material

Cysts from E. multilocularis were generously given by Dr Klaus Brehm (Institut für Hygiene und Mikrobiologie der Universität Würzburg, Germany). E. multilocularis larvae, obtained from infected patients and maintained in Mongolian jirds (Meriones unguiculatus), were cultivated in vitro for 6 weeks as described previously [20]. The cysts were washed with PBS and a soluble extract from the cyst walls was obtained as described above for the ‘mother’ bovine HCW.

In vitro complement-activation assay

The human alternative complement pathway C3 convertase was formed from its purified components [45 ng of amidated C3, 70 ng of factor B and excess factor D] in the presence or absence of the samples to be tested. Incubations were performed at 37 °C for 1 h in 5 mM NaH₂PO₄/2.5 mM barbituric acid (adjusted to pH 7
with NaOH) containing 75 mM NaCl, 0.25 mM EDTA, 0.01 % (w/v) Triton X-100 and 1 mM NiCl₂. Complement activation was evaluated in terms of factor B (93 kDa) conversion into its activation products Bb (63 kDa) and Ba (30 kDa), as visualized by Western blotting. Samples were run on SDS/PAGE [8 % (w/v) acrylamide gels] and electrotransferred on to nitrocellulose membranes. For detection of factor B, blots were probed with a rabbit anti-human factor B) polyclonal antibody (Dako, Carpinteria, CA, U.S.A.) and peroxidase-conjugated goat anti-rabbit IgG (Calbiochem). Blots were developed using enhanced chemiluminescence (ECL*; Amersham Biosciences, Piscataway, NJ, U.S.A.).

NMR analyses

NMR spectra were recorded for solutions in ²H₂O at 30 °C using a Bruker Avance DXP 400 instrument (Acton, MA, U.S.A.) at 400, 100.6 and 162 MHz for ¹H, ¹³C and ³¹P respectively. ¹H, ¹³C and ³¹P chemical shifts were reported in p.p.m. using 3-trimethylsilyl propionate (δH 0.00) and acetone (δC 31.07) as internal references and phosphoric acid (δP 0.00) as the external reference. Two-dimensional [COSY and heteronuclear multiple quantum coherence spectroscopy (‘HMQC’)] experiments were performed according to standard pulse sequences available in the Brüker software.

MS analyses

Electrospray ionization MS was performed by Dr Robin Aplin (Oxford Centre for Molecular Sciences, University of Oxford, U.K.) employing a Micromass BioQ II-ZS mass spectrometer (Micromass U.K. Ltd., Altirincham, Cheshire, U.K.). Both positive- and negative-ion spectra were recorded over a mass range of 200–1200 Da, and were calibrated relative to a mixed polyethylene glycol standard. Matrix-assisted laser-desorption ionization-time-of-flight MS was carried out by Dr Rosario Durán and Dr Carlos Cerveriánsky (Lab. de Bioquímica Analítica, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay) on a Voyager DE-PRO spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) using 2,5-di-hydroxybenzoic acid as the matrix and a 337 nm nitrogen laser.

Dephosphorylation of the HCW component and analysis of the resultant product

The purified component was freeze-dried and treated with 48 % (w/v) hydrofluoric acid overnight at 4 °C. Excess hydrofluoric acid was evaporated using N₂. The product was applied on to a P2 gel-filtration column (1 cm × 60 cm; Bio-Rad, Hercules, CA, U.S.A.) in 1 % (v/v) pyridine/0.4 % (v/v) acetic acid buffer (pH 6.0). Fractions corresponding to the refractive index peaks were analysed by ¹H-NMR. The fraction containing the dephosphorylated molecule was acetylated as described previously [21] and analysed by GLC on a Hewlett Packard 6890 gas chromatograph (HP-5 capillary column; 0.25 μm × 30 m) with a gradient of 5 °C/min from 190–250 °C, and by GLC–MS on a Shimadzu QP 1100 instrument (PTE-5 column; 0.25 μm × 30 m; Supelco, Bellafonte, PA, U.S.A.) with a gradient of 5 °C/min from 80–260 °C. The identity of the dephosphorylated molecule was confirmed by GLC using co-injection of hexa-acetylated myo-inositol prepared from commercial myo-inositol as described previously [21].

TLC for high inositol polyphosphates

TLC was performed on PEI-cellulose plates, with the detection of phosphate by ammonium molybdate as described previously [22]. The detection limit of the system was 6 nmol of phosphate. Crude extracts were dialysed against water and treated with activated charcoal prior to TLC to minimize interference with the detection of inositol polyphosphates. For quantitation of IP₆, the plates were scanned and the intensity of the spots was quantitated by densitometry (integration of density over area) using Quantity One software (Bio-Rad). Different amounts (3–15 nmol) of an IP₆ standard (Sigma) were run in parallel with the samples and employed to construct linear regression curves (r² > 0.98).

Detection of E. granulosus intracellular proteins

The intracellular parasite proteins, P-29 and cyclophilin A (CyPA), were detected by Western blotting. P-29 was detected as described previously [23]. For the detection of CyPA, samples were separated by SDS/PAGE [10 % (w/v) acrylamide gel] and electrotransferred on to nitrocellulose. Blots were probed with a rabbit polyclonal antiseraum raised against CyPA from Mesocestoides corti, a related cestode; the antisera cross-reacts strongly with the homologous protein from E. granulosus. The blots were probed with peroxidase-conjugated goat anti-rabbit IgG (Calbiochem) and developed using ECL*. M. corti CyPA, known to migrate similarly to the E. granulosus protein (M. Margenat, M. Marin and C. Fernández, unpublished work) was included as a positive control.

Quantitation of calcium and magnesium in the extracts

The quantitation of calcium and magnesium in extracts from murine cysts was carried out by Lourdes Salvarrey and D. Moises Knochen (Catedra de Analisis Instrumental, Montevideo, Uruguay) by using a PerkinElmer 380 atomic absorption spectrophotometer (Indianapolis, IN, U.S.A.). Samples were diluted in 0.5 % (w/v) lanthanum in order to avoid interference of phosphates in the determination of calcium and were subsequently burnt in an acetylene/air flame. The measurements were made using a hollow cathode lamp for calcium/magnesium/aluminium (PerkinElmer) at 20 mA, and wavelengths of 285.2 and 422.7 nm for magnesium and calcium respectively.

Quantitation of total phosphorus in the HCW

‘Daughter’ bovine hydatid cysts were washed extensively with Milli Q water, cut into small pieces, and the content of phosphorus was determined as described previously [24]. Results were expressed as the means of four individual determinations.

RESULTS

Purification and identification of IP₆ from the HCW of E. granulosus

A component, subsequently identified in the present study as IP₆, was detected in and purified from the HCW of E. granulosus as part of a search for host complement-inhibitory activities from the parasite [17]. Thus detection of the component, both in crude extracts from the HCW and during its purification, was carried out by means of an assay for inhibition of complement activation. Details of the analysis of the interaction of IP₆ with the complement system will be reported elsewhere (F. Iriogoin, A. Laich, A. M. Ferreira, C. Fernández, R. B. Sim and A. Díaz, unpublished work). The complement-inhibitory activity was not solubilized from the HCW in the absence of EDTA, suggesting that the active components were immobilized in the...
A molecule, subsequently identified as IP₆, was purified from a soluble extract of bovine HCW on the basis of its ability to interfere with complement activation. Complement activation was measured in terms of the conversion of factor B (93 kDa) into its activation fragments, Bb (63 kDa) and Ba (30 kDa), as detected by Western blotting. Hence, inhibitory activity was visualized as the absence of the bands corresponding to Bb and Ba. The results of the complement activation assay for the selected fractions are shown in the Figure insets. (A) Anion exchange on a 25 ml Source 15Q column (BioPilot; Amersham Biosciences) at pH 8.0. The elution was performed with increasing concentrations of NaCl and the absorbance (shown as optical density, O.D.) was measured at 280 nm. No absorbance at 280 nm was detected with the material that did not bind to the column. The inhibitory activity eluted at 0.25–0.4 M NaCl. (B) Active fractions from the anion-exchange step were pooled, dialysed against a solution containing 10 mM sodium phosphate buffer (pH 7.0), 250 mM NaCl and 0.5 mM EDTA and refractionated by FPLC-gel filtration on Superdex 75 (Amersham Biosciences). Absorbance (O.D.) of the eluted proteins was measured at 280 nm. Elution volumes of protein standards (in kDa) are indicated above the panel. Solid bars in both (A) and (B) represent the fractions pooled for further purification or characterization. The concentration of IP₆ in the active fractions from the second step was 3–5 mg/ml.

**Figure 2** Purification of a component of the HCW of E. granulosus with the capacity to inhibit complement activation

The purified fraction obtained was analysed by NMR, MS and TLC. The one-dimensional ¹H-NMR spectra (results not shown) and ⁴⁰P-NMR spectra (four signals at 2.95, 1.95, 0.95 and 0.05 p.p.m. with an intensity ratio of 1:2:2:1 respectively, results not shown), as well as the two-dimensional ¹H-¹H-, ¹H-¹³C- and ¹H-²⁰P-NMR spectra (results not shown), were compatible with a pure small molecule containing six carbon atoms, none of them anomeric in nature, and each bound to a phosphate group. The molecule was subjected to hydrolysis in the presence of hydrofluoric acid, with the aim of removing the phosphate groups, and the resulting product was analysed by NMR (one-dimensional ¹H- and ¹³C-NMR and two-dimensional ¹H-¹H- and ¹H-¹³C-NMR). The results suggested that the dephosphorylated molecule was myo-inositol; this was confirmed by GLC with co-injection of the acetylated derivative of commercial myo-inositol (results not shown). Electrospray ionization and matrix-assisted laser-desorption ionization–time-of-flight MS of the purified untreated molecule indicated a molecular mass of 660 Da (results not shown), which was compatible with an inositol ring with six phosphomonoester groups attached. Confirmation of the identity of the purified molecule was achieved by NMR. The ¹H- (Figure 3A) and ¹³C- (results not shown) NMR spectra of the molecule were identical with those of commercial IP₆, with a minor contaminant migrating consistently with IP₆ (Figure 3B).

**Figure 3** Identification of the purified component of the cyst wall as IP₆

The purified component of the cyst wall was analysed in parallel with IP₅ and IP₆ standards. (A) Commercial IP₅ (broken line) and the purified component (solid line) were passed through a Dowex 50 resin (H⁺ form), freeze-dried and redissolved in ²H₂O for ¹H-NMR spectroscopy as described in the Materials and methods section. Chemical shifts are expressed in p.p.m. using 3-trimethylsilyl propionate (δ_; 0.00) as an internal reference. (B) The purified component and the standards were run on PEI-cellulose TLC.

IP₆ is an intrinsic component of the HCW

The parasite material from which IP₆ was isolated, namely HCW from bovine lung cysts, exists in vivo in intimate contact with...
host inflammatory cells. Host-derived molecules can find their way in vitro into the meshwork constituted by the laminated layer and be retained within it. For example, this takes place for the usually cytosolic protein annexin II [25]. Hence, it was possible that the IP$_6$ isolated from bovine HCW originated from host cells. Metabolic labelling studies are not straightforward to carry out with the cyst stage of the parasite. Thus we used an approach consisting of assessing the presence of IP$_6$ in cysts from other origins in which it is known that the contact with host inflammatory cells is minimal or nil.

One type of material analysed was cysts obtained from experimental infection in mice. These cysts grow free in the peritoneal cavity with little inflammatory reaction around them (M. Breijo, G. Anesetti, L. Martínez, R. B. Sim and A. M. Ferreira, unpublished work). The second, even ‘cleaner’, type of material used was constituted by small (‘daughter’) cysts, which arise inside larger (‘mother’) bovine ones. As host cells cannot penetrate the HCW of the ‘mother’ cyst, ‘daughter’ cysts are free of contact with host cells. Moreover, the chemical nature of IP$_6$ (charge, insolubility in the presence of calcium) is expected to preclude it from diffusing across the membrane barriers of the germinal layer and the calcium-containing cyst fluid of the ‘mother’ cyst. Extracts were made from murine and ‘daughter’ bovine cysts, and IP$_6$ was detected and quantitated in them by TLC. Of the total dry masses of HCW from murine and ‘daughter’ bovine cysts, IP$_6$ accounted for 8 and 15%, respectively (expressed in terms of its free acid form), whereas the corresponding value for ‘mother’ bovine cysts was 3%. This latter value is probably an underestimation of the actual levels of IP$_6$ in the ‘mother’ bovine HCW, as it was observed that is more difficult to solubilize IP$_6$ from these thicker cyst walls than it is from the HCW of small cysts (results not shown). Taken together, these observations confirmed that IP$_6$ is an intrinsic component of the HCW, and is therefore, in all likelihood, produced by the parasite.

IP$_6$ was not detected by TLC in other E. granulosus materials, other than the HCW. Taking into account the amounts of material loaded and the detection limit of the method, IP$_6$ in protoscoleces and adult worms constituted less than 0.09% and 0.43%, of the total dry mass respectively. Similarly, the maximum possible IP$_6$ concentration in hydatid cyst fluid would be 9 mg/ml. We also analysed the presence of IP$_6$ in the cysts from another species of Echinococcus, namely E. multilocularis. IP$_6$ was not detected in the cyst wall from this species (less than 0.7% of the starting material dry mass).

**IP$_6$ is a component of an extracellular structure: the laminated layer of the HCW**

The extracts analysed above originated from whole HCWs, with no attempt made to separate physically the germinal and laminated layers. Since IP$_6$ has been described as an intracellular cytosolic molecule [13], it could be assumed that the IP$_6$ extracted from the HCW was contained within the cells of the germinal layer. However, some observations were not consistent with this possibility, suggesting that IP$_6$ was a constituent of the laminated layer.

One of the first observations that caught our attention was the very high amounts of IP$_6$ extracted from the HCW. A calculation on the basis of the IP$_6$ content of small cysts, their diameters and an average germinal layer thickness of 10 μm, as reported for cysts in this size range [26], yielded a hypothetical intracellular IP$_6$ concentration of approx. 300 mM. This is hundreds of times higher than the concentration of IP$_6$ in Dictyostelium discoideum, one of the highest reported [27]. If present at high concentrations in the cytosol, IP$_6$ would be present (mostly in insoluble form) as its magnesium salt [6]. In contrast, extracellular IP$_6$, even at submicromolar concentrations, would be present as its calcium salt, which is even more insoluble than the magnesium one. This reasoning led us to quantitate the amounts of calcium and magnesium co-extracted with IP$_6$. As shown in Table 1, calcium and magnesium were extracted at molar ratios with respect to IP$_6$ of 8 and 0.7, respectively. These results suggested that most of the IP$_6$ is present as the Ca$_6^{2+}$ salt; assuming this is the hexacalcium salt, association with IP$_6$ would account for approx. 74% of the total calcium extracted from the HCW. These data also suggested that the majority of the IP$_6$ extracted was not present in the cytosol of cells.

We then attempted the selective stepwise extraction of IP$_6$ from the cellular and acellular layers of the structure. The pattern of extraction was then compared with those of two intracellular markers: the parasite proteins P-29 [23] and CyPA [28]. Both proteins are known to be expressed by the germinal layer cells and have relatively low molecular masses (29 and 16 kDa for P-29 and CyPA respectively). CyPAs are exclusively cytosolic proteins [29]; the subcellular location of P-29, which is absent from parasite secretions, has not been determined experimentally, but the lack of sorting signals in its coding sequence suggests that the protein is also cytosolic.

Intact murine or ‘daughter’ bovine cysts were washed with PBS and then PBS/EDTA in an attempt to extract any IP$_6$ present in the laminated layer without disrupting the integrity of the germinal layer cells. Cyst fluid was then removed by aspiration with a syringe, and the resultant HCW was opened up and extracted with PBS/EDTA/Tween in order to solubilize cellular components. Washing the intact cysts in the presence of EDTA allowed the extraction of some IP$_6$ (Figure 4A and Table 2). No IP$_6$ was recovered from the cyst fluid, but the treatment of the opened-up cyst walls with PBS/EDTA/Tween allowed the extraction of some IP$_6$ (Figure 4A). In contrast with IP$_6$, the two intracellular proteins P-29 and CyPA were only extracted when cells were disrupted with the use of detergent. No contamination with these intracellular markers was observed in the external washes of intact cysts with the EDTA-containing buffer (Figures 4B and 4C). In a parallel experiment, EDTA was injected into intact murine cysts, so as to attain the same final concentration of the chelating agent in the cyst fluid as that used in the external washings. This extraction of the HCW ‘from the inside’, did not solubilize any IP$_6$ (Figure 4A). This indicated that IP$_6$, solubilized during the external washings of the cysts, could not have arisen from the disruption of cells as a result of the chelation of bivalent cations by EDTA.

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**Table 1 Quantitation of IP$_6$, calcium and magnesium in different extracts from murine hydatid cysts**

<table>
<thead>
<tr>
<th>Extract</th>
<th>IP$_6$</th>
<th>Calcium</th>
<th>Magnesium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washes from outside</td>
<td>101</td>
<td>804</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(0.8)</td>
<td>(0.6)</td>
</tr>
<tr>
<td>Opened-up cysts</td>
<td>22</td>
<td>200</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(1.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>123</td>
<td>1004</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(0.7)</td>
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or PBS/EDTA (EDTA) respectively. PBS, the germinal and laminated layers were extracted with PBS/EDTA/Tween (Tween-EDTA) subjected to mechanical separation of its constituent layers using forceps. After washing with to that used for washing the intact cysts ‘from the outside’. After incubation, the cyst fluid from inside) by injecting the chelating agent, so as to attain an intracystic concentration equal experiment, murine cysts (6 cysts) were extracted with EDTA ‘from the inside’ (whole cysts opened up (opened-up cysts) and extracted with PBS/EDTA/Tween (Tween-EDTA). In a parallel these extracts are grouped under ‘whole cysts from outside’. After cyst fluid removal, HCW were left panel) cysts (11 and 9 cysts respectively) were successively washed with PBS (no EDTA) and PBS/EDTA (EDTA). An extra PBS wash was then performed on the murine cysts. All of intracellular proteins P-29 and CyPA. Intact murine (upper panel) and ‘daughter’ bovine (lower, E. granulosus protein, was included as a positive control. When sequential extracts from the same DISCUSSION

In the current study, we report the presence of high amounts of IP₆ in the laminated layer of the HCW of the parasitic cestode *E. granulosus*. IP₆ was purified from a soluble extract of the HCW and was shown to account for up to 15% of the total dry mass of this structure. However, the actual amounts of IP₆ in the HCW could be higher than those estimated in the present study. Although IP₆ behaves in dialysis as if it had a higher molecular mass than predicted from its formula [10,15], it is expected that a minor percentage of the IP₆ in the samples is lost during dialysis against water prior to quantitation. In any case, this underestimation is unlikely to have been large, as the value for total phosphorus in the HCW was only slightly higher than that calculated to derive from IP₆. A previous study [30] has demonstrated strikingly high amounts of IP₆ in animal species: IP₆ insolubilized as its Mg²⁺ salt accounted for 50% of the total dry mass of the dispersal larvae of the mesozoan *Dicyema typus*. Even in that particular case, IP₆ is present intracellularly. In contrast, the majority of the IP₆ in the HCW of *E. granulosus* occurs in the acellular laminated layer. The secretion of endogenously synthesized IP₆ by cells has not been reported to date, causing the various extracellular activities observed for IP₆.
to be regarded as unlikely to be of physiological importance [5]. Our findings constitute the first observation of IP₄ being an intrinsic component of an extracellular structure.

The building blocks of the laminated layer are synthesized and secreted by the cells of the germinal layer. The possibility of IP₄ being accumulated in the laminated layer by other means, i.e. as a result of necrosis of germinal layer cells, seems remote. Firstly, there is no evidence of extensive necrosis of germinal layer cells. Secondly, if we assume that the IP₄ concentration in the cells of the germinal layer were as high as in D. discoideum, i.e. about 1 mM [27], the whole germinal layer would have to release its contents 200 times over for the amount of IP₄ found in the laminated layer to be attained. Thus IP₄ has to be synthesized by the cells of the germinal layer and subsequently secreted, in a polarized fashion, into the extracellular environment. If the synthesis of IP₄ in E. granulosus cells takes place in the cytosol, as it is the case in unicellular eukaryotes for which the synthetic pathway of IP₄ has been elucidated [31,32], IP₄ would have to cross a membrane barrier at some point along its secretion pathway. This would imply the presence of a membrane translocator for IP₄. The existence of such a transporter in the membranes of the ER of mammalian cells has been suggested to explain the observation that multiple inositol polyphosphate phosphatase, a phosphatase present in the lumen of the ER, affects the cytosolic levels of IP₃ [16]. As the E. granulosus germinal layer must have a very active machinery to synthesize and secrete IP₄, this constitutes an interesting system for studying the metabolism and possible membrane translocation of this molecule.

IP₄ in the laminated layer is immobilized by the interaction with Ca²⁺, as it was solubilized only in the presence of EDTA, co-solubilizing with Ca²⁺ and not with Mg²⁺. Chemical analyses of the laminated layer have shown that the structure is much richer in Ca²⁺ than in Mg²⁺ [33]. It is likely that IP₄ is involved in this selective retention of Ca²⁺. Ca²⁺ deposits in the laminated layer have been ascribed by X-ray microanalysis to the naturally electron-dense aggregates, which are one of the main ultrastructural features of the structure [34]. The aggregates are found irregularly dispersed within the microfibrillar matrix, the major ultrastructural element of the laminated layer. This organization has been observed consistently in E. granulosus HCDs from various origins [26]. The microfibrillar matrix is a common feature of the laminated layers of different Echinococcus species [35,36]. In contrast, the naturally electron-dense aggregates have only been described in E. granulosus [37]. The structures of these aggregates have been described in detail: they are composed of granules that are homogeneous in size (41 ± 1 nm) and possess 8 nm electron-lucent spheres within. The granules are also present within vesicles in cells of the germinal layer, which appear to arise from the Golgi apparatus and to be involved in exocytosis towards the laminated layer. It seems likely that these Ca²⁺-containing granules contain the IP₄ present in the HCW. We are currently testing this hypothesis.

The biological function of IP₄ in the E. granulosus laminated layer remains to be answered. Although a strictly structural role is plausible, the large extracellular amounts of the molecule found specifically at the interface with the host suggest a function related to the interaction with host molecules. IP₄ would provide the laminated layer, which is a structure composed mainly of neutral sugars [38], with charge. It is well established that certain extracellular cascades of the mammalian host, namely the alternative and classical pathways of complement and the intrinsic pathway of coagulation, are sensitive to distributions of charge present or absent on surfaces [39,40]. There have been several reports on the selective adsorption of host proteins in the E. granulosus HCW. These proteins, namely factor H [41], annexin II [25], calphin K [42] and the extracellular superoxide dismutase (G. Salinas, unpublished work), share the capacity to bind to negatively charged species, often glycosaminoglycans (GAGs). GAGs could not be detected in the HCW by histochemical techniques (A. Díaz and R. B. Sim, unpublished work). The possibility that IP₄ is responsible for the retention of GAG binding host proteins in the HCW deserves further study. Finally, the fact that IP₄ does not appear to be a major component of the cyst wall of E. multilocularis needs to be taken into account when considering the function of the compound in E. granulosus. Although in both species the laminated layer protects the parasite from host immune cells, several other features of the host–parasite relationships established by the two are very different. Whereas the unilocular, non-invasive cyst of E. granulosus manages not to trigger inflammation in most host species [43], E. multilocularis displays a tumour-like growth, with external cyst budding and invasion of the host organ parenchyma, leading to necrosis, chronic granulomatous inflammation and extensive fibrosis [44].

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