Ultrastructural localization of an *Echinococcus granulosus* laminin-binding protein

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SUMMARY

Murine antibodies, raised against a purified recombinant 30 kDa laminin-binding protein from *Echinococcus granulosus*, were used to investigate the tissue distribution of the native protein in protoscoleces and brood capsules. Immunofluorescence, in combination with confocal microscopy, revealed that the protein was distributed in small annular foci near the peripheral regions of the protoscoleces. Immunoelectron microscopy of thawed cryosections demonstrated that the laminin-binding protein was present in the cytoplasm of tegumentary cytons and myocytons, but not in cells of the excretory system. The protein was associated with amorphous regions of the cytoplasm, and was not expressed at the surfaces of cells. This distribution resembles those of other invertebrate laminin-binding proteins, which are thought to act in the cell cycle and cell proliferation events. A low degree of label was consistently detected in extracellular matrices of the protoscolex.

Key words: Cestoda, *Echinococcus granulosus*, laminin, laminin-binding protein, ribosomal protein, immunolocalization, ultrastructure, tegument.

INTRODUCTION

A cDNA (egmo3) from the hydatid tapeworm, *Echinococcus granulosus*, encoding a 30 kDa protein has been isolated recently (Zhang *et al*. 1997). The cDNA clone shows substantial homology with a non-integrin mammalian metastasis-associated 67 kDa laminin receptor (67-LR) (Castronovo, 1993) as well as the acidic class of ribosomal proteins (Zhang *et al*. 1997). *In vitro* binding assays indicate that the recombinant protein and 1 subunit encoded by the egmo3 clone can bind laminin (Zhang *et al*. 1997). It is thought, therefore, that the *E. granulosus* laminin-binding protein may be multifunctional (Zhang *et al*. 1997) and plays a role in cellular differentiation as well as cell–extracellular matrix interactions.

The mammalian 67-LR is thought to play a crucial role in cancer metastasis; this molecule and its precursors are upregulated in aggressive carcinomas and the protein promotes binding of the tumours to parenchyma (Clausse *et al*. 1996; Tuszynski, Wang & Berger, 1997; Yam *et al*. 1997). However, earlier work suggested that mammalian laminin-binding protein acts as a cofactor in translational control (Grosso, Park & Mecham, 1991). Invertebrate homologues of the 67-LR have been described for members of the phyla Echiura, Cnidaria and Platyhelminthes (Rosenthal & Wordeman, 1995; Keppel, Fenger & Schaller, 1997; Zhang *et al*. 1997) and a molecule sharing immunological identity with the 67-LR has been demonstrated in 1 species of pathogenic yeast (McMahon *et al*. 1995). The 67-LR-like molecules of *Urechis* and *Hydra* appear to function in cell cycle and cell proliferative events and not as laminin receptors (Rosenthal & Wordeman, 1995; Keppel *et al*. 1997).

The *E. granulosus* hydatid cyst is a complex structure, composed of tissues with a seemingly unlimited proliferative potential (reviewed by Thompson, 1995). The tissue of the *E. granulosus* protoscolex consists of cells embedded in an extensive extracellular matrix (ECM). Extensive interactions between cells and ECMs are likely to occur, particularly during differentiation of the parasite. It is of interest, therefore, to determine whether the *E. granulosus* laminin-binding protein (LBP) functions in cell proliferation events or in cell–ECM interactions.

Here, we have used a murine polyclonal antiserum directed against the recombinant *E. granulosus* LBP (Zhang *et al*. 1997) in an immunocytochemical study to define the subcellular location of the protein in protoscoleces and brood capsules to help determine biological roles of this molecule.

MATERIALS AND METHODS

Parasites

Protoscoleces and brood capsules of *E. granulosus*

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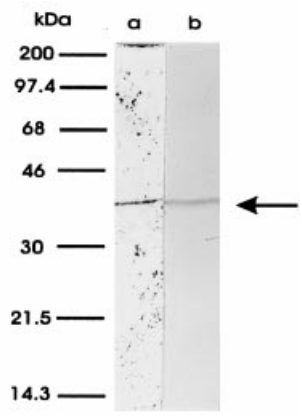

Fig. 1. SDS–PAGE and immunoblot analysis showing the molecular mass (approximately 37 kDa) and purity of the recombinant *Echinococcus granulosus* LBP, and the specificity of murine antibodies raised to it. The purified, recombinant protein (2 mg) was subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose and silver stained (lane a). Its position (arrowed) and M_r were confirmed by Western blot using the murine anti-*E. granulosus* LBP antiserum and goat anti-mouse IgG horse-radish peroxidase (Sigma) conjugate (lane b). The absolute specificity of the murine antiserum was confirmed in a parallel immunoblot where the anti-*E. granulosus* LBP antibodies were shown to bind a single native protein in *E. granulosus* protoscoleces (see Fig. 7, Zhang *et al.* 1997).

were obtained from cysts of sheep origin in Australia and China. For immunofluorescence, specimens were fixed in methanol at −20 °C. For ultrastructural localization studies, specimens were fixed in 4% paraformaldehyde in 0·1 M cacodylate buffer, pH 7·2, for 2 h and washed in 3 changes of cacodylate buffer.

Production of the murine polyclonal antiserum directed against the E. granulosus *LBP*

The murine polyclonal antiserum was prepared and tested for specificity as described by Zhang *et al.* (1997). Briefly, the cDNA encoding the *E. granulosus* LBP was subcloned into the QIA express vector, pQE31 and the 6XHis fusion protein expressed in *Escherichia coli* SURE cells. The recombinant protein was purified by FPLC under denaturing conditions, dialysed against several changes of PBS and then used to immunize mice in combination with Freund's adjuvant. The specificity of the resultant antibodies was confirmed by reducing SDS–PAGE and immunoblot using both the recombinant protein and intact, whole *E. granulosus* protoscoleces as target antigen sources.

Immunofluorescence and immunocytochemistry

Methanol- and paraformaldehyde-fixed worms were

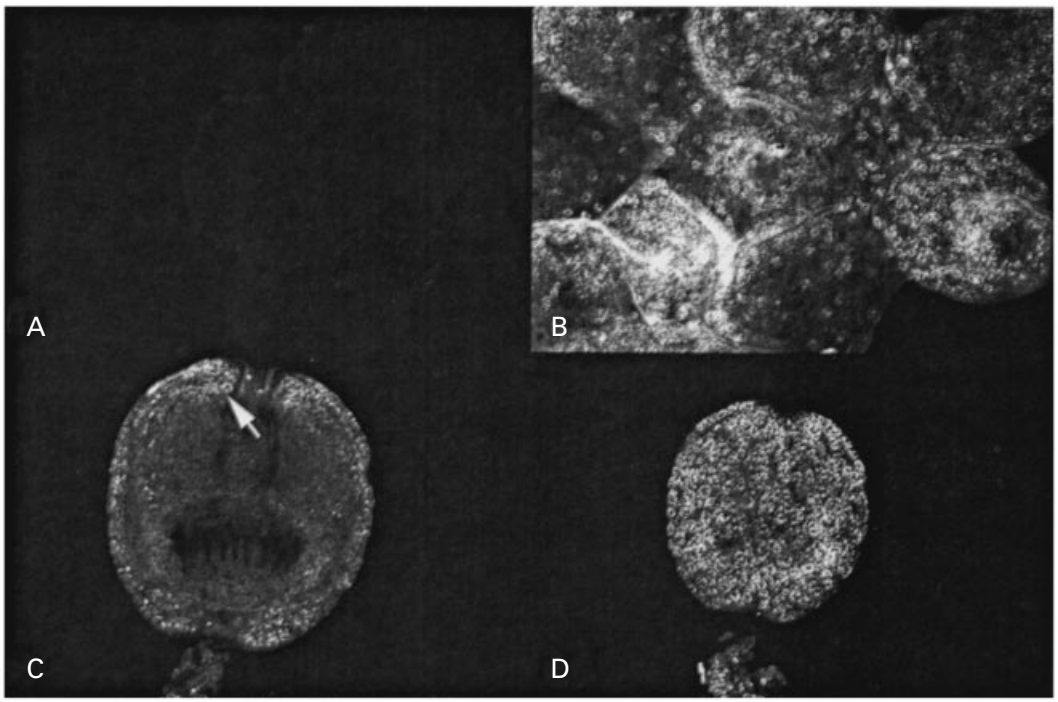

Fig. 2. Immunolocalization of *Echinococcus granulosus* LBP, confocal images. (A) Negative control. (B) Protoscoleces in brood capsule. Note the ring-shaped pattern of labelling in some protoscoleces. (C) Protoscolex, optical sections through the centre of the worm. Most immunoreactive material is evident in peripheral regions. Intense immunoreactivity is evident anteriorly, at the point of invagination of the scolex (arrow). (D) The same protoscolex as in (C), peripheral region. Note the strong labelling pattern, which is evident in a ring-shaped pattern.

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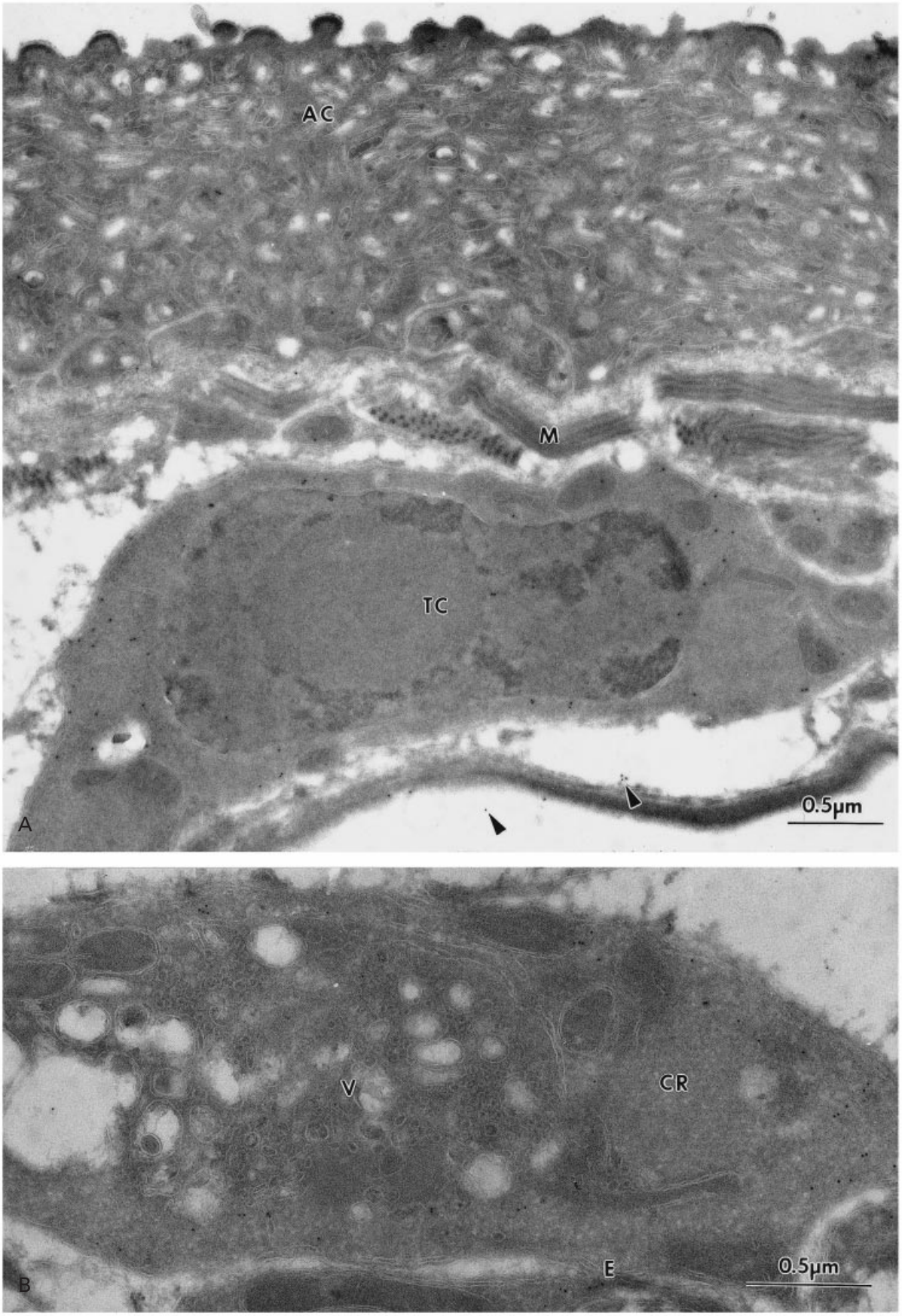

Fig. 3. Immunolocalization of *Echinococcus granulosus* LBP, TEM. (A) Somal tegument and tegumentary cyton. The protein is evident in the cytoplasm of a tegumentary cyton, but not the apical cytoplasm, or underlying musculature. Some label is evident in the extracellular matrix (arrowhead). (B) Tegumentary cyton. Note the vesicles and crystalloid arrays. Immunoreactivity is confined to a thin cytoplasmic rim. AC, apical cytoplasm of the tegument; CR, crystalloid array; E, endoplasmic reticulum; M, myofibril; TC, tegumentary cyton; V, tegumentary vesicles.

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washed in phosphate-buffered saline (PBS) supplemented with 0·2% (v/v) Triton X–100 (PBT) and incubated in primary antiserum diluted 1:50 or 1:200 in PBT for 24 h. After washes in PBT, samples were incubated in CY-3-labelled goat anti-mouse IgG (Jackson, Australia) diluted 1:500 in PBT for 12–24 h. Further washing in PBT and PBS was performed and the samples were mounted on glass slides for fluorescence and confocal microscopy. Samples were examined by confocal microscopy using a Bio-Rad MRC 600 fitted with a Krypton/Argon laser and a Zeiss Axiophot microscope or by fluorescence microscopy using an Olympus BX-60 fluorescence microscope. The plate was constructed from grey-scale confocal images using Adobe Photoshop Version 4.0 (Adobe Systems Inc, USA).

On-grid labelling of cryosections followed methods outlined previously (Jones *et al*. 1996). Briefly, thawed cryosections on formvar-carbon coated copper grids were incubated on droplets containing the following solutions (i) 0·2 M (w/v) glycine in PBS, (ii) PBS, (iii) 1% (w/v) bovine serum albumin (Fraction V, Sigma) in PBS (BSA/PBS), (iv) anti-LBP serum diluted 1:100 to 1:300 in BSA/PBS, (v) 4 changes of BSA/PBS and (vi) goat-anti-rabbit IgG conjugated to 10 nm gold particles (Biocell, Cardiff, UK) in BSA/PBS. Subsequently, grids were rinsed in 2 changes each of BSA/PBS and PBS, followed by postfixation of sections in 1% glutaraldehyde (v/v) in PBS and washes in changes of PBS, and distilled water. Sections were contrasted and dried in 0·4% uranyl acetate in 1% aqueous methyl cellulose and examined using a JEM 1010 transmission electron microscope at 80 kV. Controls used for immunocytochemistry included pre-immune mouse serum diluted at 1:100 to 1:300 and omission of the primary antiserum from incubation steps.

RESULTS

Specificity of the murine polyclonal antibodies directed against the E. granulosus *LBP*

Reducing SDS–PAGE/silver staining of the recombinant *E. granulosus* LBP indicated the protein had been purified to homogeneity (Fig. 1). The absolute specificity of murine antibodies raised to the purified protein was confirmed by immunoblot using as target antigen source both the recombinant protein (Fig. 1) and whole *E. granulosus* protoscoleces (see Fig. 7 in Zhang *et al*. 1997).

Immunofluorescence and confocal microscopy

By immunofluorescence, the *E. granulsous* LBP was detected in protoscoleces, brood capsule tissues and peduncles (Fig. 2B–D) in methanol- and formalin-fixed tissues. Our immunolabelling protocol worked more readily on methanol-fixed tissues, possibly because the tissues were better permeabilized by this fixative. Omission of the primary antiserum from the immunofluorescence assay failed to produce any immunoreactivity (Fig. 2A). The *E. granulosus* LBP was distributed in small annular foci (Fig. 2B–D) suggesting a cytoplasmic location for this protein. The immunoreactive cells were abundant in peripheral regions of protoscolex tissues (Fig. 2C, D) and the subepithelial layers of the brood capsules and peduncles (not figured). Comparatively little immunoreactivity was evident in deeper regions of the invaginated protoscoleces (Fig. 2C). There was intense reactivity at the point of invagination of the scolex, which is the region where stem cells are likely to occur.

Immunoelectron microscopy

Sections were immunolabelled with primary antiserum diluted at 1:100 to 1:300 in BSA/PBS. The different dilutions of serum produced essentially the same labelling patterns, although non-specific labelling of support film and tissues was higher at higher serum concentrations. No gold label was detected on sections for which pre-immune mouse serum was used or for which the primary antiserum was omitted from the labelling protocol (data not shown).

Label was detected in 2 primary tissue compartments of the protoscolex, namely, extracellular matrix and cytoplasmic domains of some cells. The protein was not observed in the tegumental lining of the soma or the scolex region (Fig. 3A), or in excretory ducts and their associated cell bodies. No label was detected in the portions of brood capsules we examined by electron microscopy.

Labelling in the extracellular matrix occurred over amorphous and lucent regions of the protoscoleces (Figs 3A and 4A). This extracellular label was present in all experimental sections regardless of antibody dilution, but the degree of labelling in the ECM was always lower than that of the cytoplasmic labelling.

Cytoplasmic labelling was detected in subtegumental cytons of the soma (Figs 3A, B and 4A), and in myocytons (Fig. 4B) occurring among the myofibril-rich tissues beneath the invaginated scolex. Tegumentary cytons (Figs 3A, B and 4A)

Fig. 4. Immunolocalization of *Echinococcus granulosus* LBP, TEM. (A) Tegumentary cyton. The laminin binding protein is restricted to the apparently amorphous regions of cytoplasm and is not present among the tegumentary vesicles. Some label is evident in extracellular matrices. (B) Myocyton. CR, crystalloid array; E, endoplasmic reticulum; M, myofibril; V, tegumentary vesicles.

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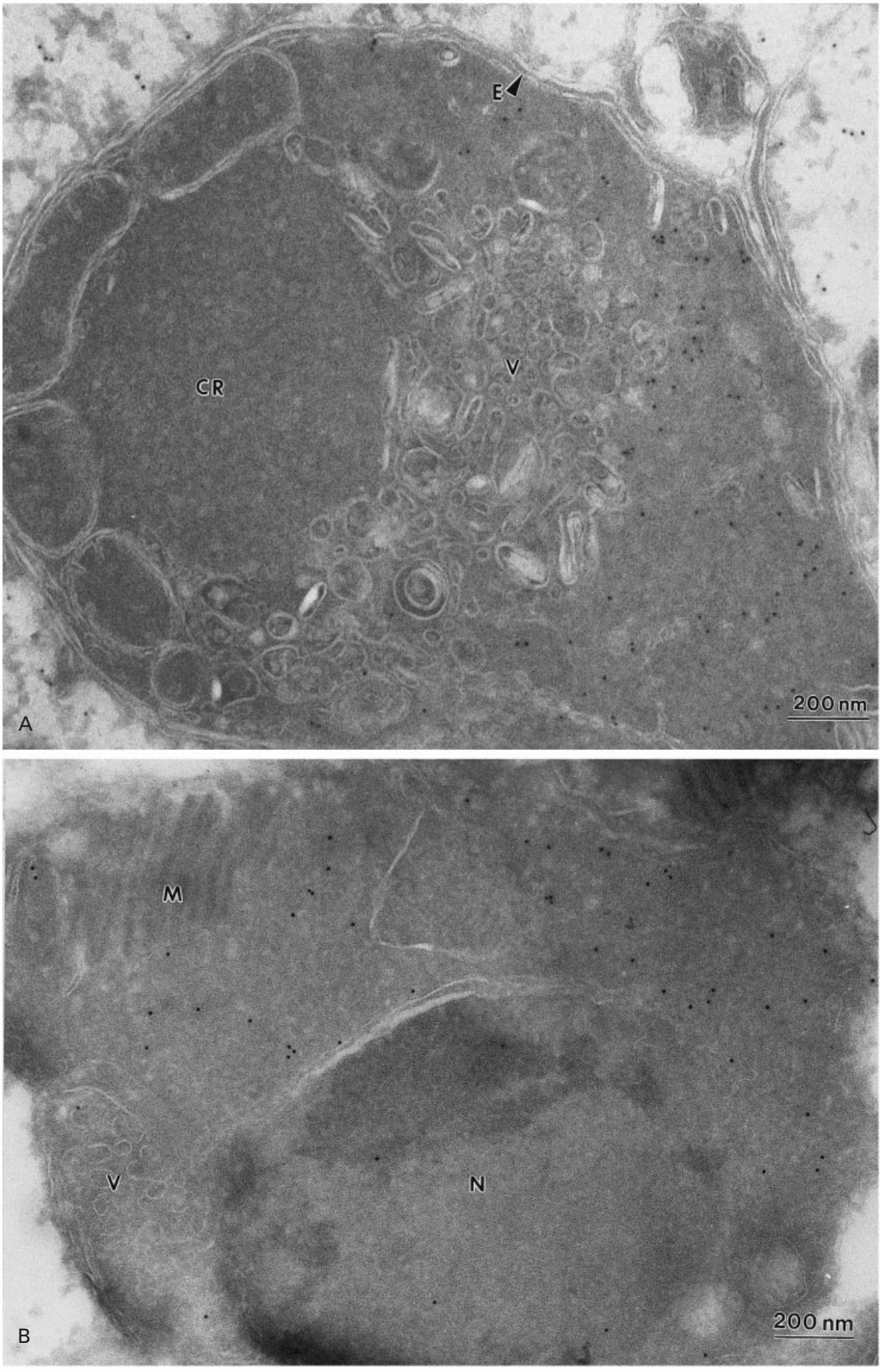

Fig. 4. For legend see opposite.

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cells were particularly abundant beneath the somal tegument and were flask-shaped. The cytons are characterized by a moderate nucleus-to-cytoplasmic ratio and the occurrence of abundant tegumentary vesicles, perinuclear crystalloid arrays (Morseth, 1967) and peripheral arrays of endoplasmic reticulum (Figs 3A, B and 4A). The *E. granulosus* LBP was detected in cytoplasmic regions of these cell bodies (Figs 3A, B and 4A). No label was detected over prominent organelles (Figs 3B and 4A), such as tegumentary vesicles or crystalloid arrays. The protein did not appear to be expressed on the surface of cells, although there was some evidence of labelling in the apical arrays of endoplasmic reticulum.

LBP was detected in amorphous cytoplasmic regions of myocytons. These cells were characterized by a large nucleus to cytoplasmic ratio, and few cytoplasmic organelles, although a peripheral ring of endoplasmic reticulum was evident (Fig. 4B). The cells were observed beneath the tegument of the invaginated scolex. Most of these cells were elongate and some were in cytoplasmic continuity with myofibrils (Fig. 4B).

DISCUSSION

Immunofluorescence microscopy of the *E. granulosus* LBP demonstrated that the molecule is distributed in an annular pattern primarily in peripheral regions of protoscoleces, suggesting a cytoplasmic location for the molecule. Ultrastructural localization experiments revealed a strong cytoplasmic labelling in tegumentary cytons and myocytons, thus confirming the immunofluorescence results.

Some label was evident in extracellular locations, but it was almost invariably located in amorphous, electron-lucent regions. The significance of this extracellular labelling is difficult to interpret. Cestodes have extensive extracellular matrices and these contain laminin fibrillar components (reviewed by Lumsden & Specian, 1980; Conn, 1993). It might be expected that a laminin-binding molecule would occur among extracellular matrices or, at least, at the cell surface. Cestode extracellular matrices can be electron lucent, with little material resolvable by conventional electron microscopy techniques (Conn & Rocco, 1989; Conn, 1993). Therefore, it is possible that the antiserum used here is demonstrating extracellular sites of the LBP. However, in the absence of any recognizable components labelled the possibility that this labelling is background cannot be excluded. On the other hand, cytoplasmic labelling of the *E. granulosus* LBP was consistently strong and localized in well-defined regions of the cytoplasm. These observations strongly implicate the molecule as a cytoplasmic constituent in the protoscolex and brood capsules.

The mammalian 67-LR is expressed on the surface of cells and its expression increases in carcinomas (Sanjuan *et al*. 1996). The surface location of 67-LR is thought to aid the cancer cells in metastasis (Tuszynski *et al*. 1997). The *E. granulosus* LBP is not expressed on cell surfaces in mature protoscoleces and, indeed, is not expressed on any external surfaces of the protoscoleces or brood capsules. Hence, it would appear that in these developmental stages, at least, the protein does not play a significant role in cell–extracellular matrix interactions. The possibility that *E. granulosus* LBP is expressed on the surfaces of other external epithelia, such as those of the oncosphere or laminated layers of the hydatid cyst, cannot be excluded until the distribution of the LBP in these tissues is investigated.

Rosenthal & Wordeman (1995) presented data to suggest that the LBP/p40 of *Urechis caupo*, a protein sharing substantial identity with human 67-LR, was associated with polysomes or free ribosomal subunits in a variety of cells of this echiurid. Although these authors could not preclude the role of laminin binding for the LBP/p40 protein, they argued that the molecule was most likely to be associated with protein synthesis. Keppel and colleagues (1997) described features of a protein from *Hydra* that resembled the mammalian LBP. This hydrozoan protein was highly expressed in cells with a short cell-cycle but not in fully differentiated cells. *In vitro* studies indicated that the hydrozoan LBP is a substrate for cell-cycle specific kinase CDC2 suggesting a role for the molecule in cell differentiation (Keppel *et al*. 1997).

Our immunocytochemical data suggest that the *E. granulosus* LBP is primarily an internal protein of cells in protoscoleces. Tegumentary cytons and myocytons can be characterized by abundant free ribosomes (Lumsden & Specian, 1980) and the molecule may be associated with these structures. It is possible also that the 30 kDa *E. granulosus* LBP resembles other invertebrate laminin-binding proteins rather than mammalian laminin receptors. Nevertheless, the recombinant *E. granulosus* LBP is capable of binding laminin (Zhang *et al*. 1997). Clearly, the precise function of the *E. granulosus* LBP requires further investigation. In particular, it would be instructive to examine the distribution of the protein in key developmental stages, such as activated protoscoleces and the proliferative neck region of adults.

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