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Cytokeratin and Desmoplakin Analogues within an Intracellular Parasite

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Abstract. A significant amount of the total protein in the spore sacs of the microsporidian *Thelohania* sp. consisted of the cytoskeletal elements, cytokeratin intermediate filaments, and the desmosomal analogues. The cytokeratin and desmosomal analogues were organized as cage envelopes surrounding the spores within the spore sac stage. *Thelohania* sp. parasitizes the skeletal muscle of *Callinectes sapidus,* a crustacean that does not appear to have cytokeratins or desmosomes. Immunoprobe data indicate *Thelohania* sp. has a 240 kDa desmoplakin protein and 48, 51, 54 and 56 kDa cytokeratin polypeptides responsive to antibodies developed against bovine cytoskeletal counterparts. The cytoskeletal envelopes within the *Thelohania* sp. spore sac stage appear to enhance the stability and viability of the spores.

Introduction

Cytokeratin intermediate filaments and desmosomal proteins are specific markers for epithelial cells in vertebrates (Cowin *et al.,* 1985; Romano *et al.,* 1986). Using high resolution two-dimensional gel electrophoresis, over 15 distinct cytokeratin polypeptides have been characterized from vertebrates (Cooper *et al.,* 1984). Monoclonal antibody studies indicate that some of these cytokeratins have analogues present in lower vertebrates (Rungger-Brandle *et al.,* 1989). Indeed, antibody cross-reactivity studies indicate that intermediate filament (IF) epitopes are shared among the different IF families; this cross-reactivity extends to IFs present in many invertebrate groups (Bartnik and Weber, 1989). However, there is no evidence that a monoclonal antibody, directed to mammalian cytokeratin, cross-reacts with presumptive cytokeratin counterparts present in epithelial cells of invertebrates (Fuchs and Marchuk, 1983; Weidner, unpub. data).

The desmosomal plaque elements consist of desmoplakin I (240–250 kDa), a protein that localizes to the region of the desmosomal plaque where cytokeratin binds (Jones and Goldman, 1985). Desmoplakin antibody shows cross-reactivity to this protein in epithelia from various vertebrate groups (Rungger-Brandle *et al.,* 1989). While desmosome assemblages and cytokeratin-like IFs are apparent in invertebrate epithelial cells, these proteins are reported to be absent in arthropods (Bartnik and Weber, 1989). The absence of these proteins in arthropods is noteworthy because we report here that cytokeratin and desmoplakin analogues are present in an intracellular parasite found within arthropods. The cytokeratin and desmoplakin analogues present in the microsporidian parasites cross-react with monoclonal antibodies directed to mammalian cytokeratins and desmoplakin.

Materials and Methods

Animal and cell preparations

Thelohania sp. was taken from blue crabs (*Callinectes sapidus*) collected from Mississippi Sound and the west coast of Florida near St. Petersburg. After removing the *Thelohania* sp.-infected muscle, the dissociated infected muscle fibers were applied to glass slides and fixed, permeabilized in 100% methanol, and further processed for immunofluorescence microscopy. Other infected muscle was washed in 0.5 m*M* $CaCl_2$, and the *Thelohania* sp. spore sac stage was liberated and purified into populations

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of spore sacs following a wash cycle described elsewhere (Weidner, 1976).

Electron microscopy

Infected skeletal muscle with *Thelohania* sp. spore sac stages from the blue crab, *Callinectes sapidus,* were prepared so that the stages of spore sac development could be examined. Also, isolated spore sacs were fixed, washed, embedded, and processed for electron microscopy as described elsewhere (Overstreet and Weidner, 1974).

Antibodies

The following primary antibodies were used: mouse cytokeratin antibody clones Lu5, AE1 and 3 (Boehringer Mannheim, Indianapolis, Indiana), K8.12, K8.13, DK802 (Sigma Chemical Co., St. Louis, Missouri), and mouse desmoplakin antibody clones PD2.15 and PD2.17 (ICN, Costa Mesa, California). Whereas, clones K8.12, K8.13, Lu5 and AE1 and 3 react to a number of epitopes common to cytokeratins, DK802 has affinity for desmosomal-binding cytokeratin 8. Second antibodies were rabbit immunoglobulins against mouse immunoglobulins coupled to alkaline phosphatase, FITC, or peroxidase (Sigma Chemical).

Immunofluorescence and immuno-electron microscopy

Infected blue crab muscle fibers or isolated *Thelohania* sp. spore sacs were fixed and permeabilized in 100% methanol and processed for indirect immunofluorescence as described elsewhere (Pasdar and Nelson, 1988). Cells were washed in PBS and incubated with anti-desmoplakin or anti-cytokeratin diluted 1:100 with PBS for 30 min at 37°C. After five washes in PBS, cells labeled with anti-mouse immunoglobulin coupled to FITC (Sigma Chemical). After five washes with PBS, cells were mounted in 20% glycerol and viewed with a 60× objective on a Nikon Microphot FXA equipped with epifluorescence illumination; images were recorded on Tri-X film (Eastman Kodak, Rochester, New York). For immuno-electron microscopy, cells were permeabilized with methanol and further fixed in 1% glutaraldehyde for 20 min. Cells were then washed in cacodylate buffer, transferred to PBS, and later immersed into primary antibody in PBS for 1 h. After 30 min of repeated washings in PBS, cells were exposed to anti-mouse conjugated to peroxidase (Sigma Chemical) for 30 min, washed in PBS, and exposed to diaminobenzidine working medium and processed for electron microscopy as described earlier (Pleshinger and Weidner, 1985).

Gel electrophoresis of Thelohania *sp. spore sac proteins and immunoblotting*

Spore sacs were liberated from *Thelohania* sp.-infected blue crab muscle with a glass homogenizer. The cell suspension was placed in 0.5 m*M* $CaCl_2$ washed and processed to purification with the wash cycle described earlier (Weidner, 1976). The spore sac desmosomal analogues were disrupted by immersing the sacs in 0.5 m*M* EGTA for 30 min. Protein samples were prepared by homogenization in boiling SDS sample buffer. The discontinuous buffer system of Laemmli (1970) was used in polyacrylamide gradient gels (7.5–15%). Samples with 15–20 μg protein per lane were heated for 5 min with 2% SDS and 3% 2-mercaptoethanol before loading for electrophoresis. Proteins were stained with Coomassie blue. Proteins for duplicate unstained gels were transferred electrophoretically to nitrocellulose membrane using a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Palo Alto, California) overnight at 4°C 50V in Tris-glycine buffer, pH 7.5 with 20% methanol. The nitrocellulose was treated with blocking buffer (1% milk powder, 0.02% Tween 20, 0.02% sodium azide in PBS) for 3 h before incubating in primary antibody (1:100 dilution in blocking buffer overnight). The nitrocellulose membrane was washed five times (10 min/wash) in PBS and transferred to anti-mouse coupled to alkaline phosphatase (1:100 dilution in blocking buffer) for 6 h. The nitrocellulose membrane was then washed five times (10 min/wash) in PBS and the antibody was visualized in incubating nitrocellulose in alkaline phosphatase substrate (100 m*M* Tris, pH 8.8, 0.01% nitroblue tetrazolium and 0.005% 5 bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical).

Results

A large percentage of the microsporidian parasites develop a sporophorous vesicle (spore sac) stage that surrounds the spores. Within *Thelohania* sp., the extrasporular space within the spore sac stage is filled with intermediate filaments (IFs) that appear attached to desmosomal or half-desmosomal plaques as illustrated in Figure 1A. Within *Thelohania* sp., eight spores are tightly packed within a spore sac (Fig. 1B). The spore sacs were extremely stable and resisted dissociation in dithiothreitol, 2-mercaptoethanol, SDS, 10 *M* urea, methanol, or organic solvents such as choloroform. However, *Thelohania* sp. spore sacs were partially permeabilized with 0.5 EGTA and subsequent shearing with a glass homogenizer caused up to 20% of the spores to liberate from the spore sacs as shown in Figure 1C.

Fluorescent antibody labeling for cytokeratins and desmosomal proteins showed a strong fluorescence primarily on the spore envelopes of *Thelohania* sp. Figure 2A depicts phase optical imaging of spore sacs recovered from infected muscle of *Callinectes sapidus.* On the basis of antibody labeling, only 15–20% of the spore sacs were successfully permeabilized for antibody labeling. All spores liberated from the sacs were reactive to specific antibodies

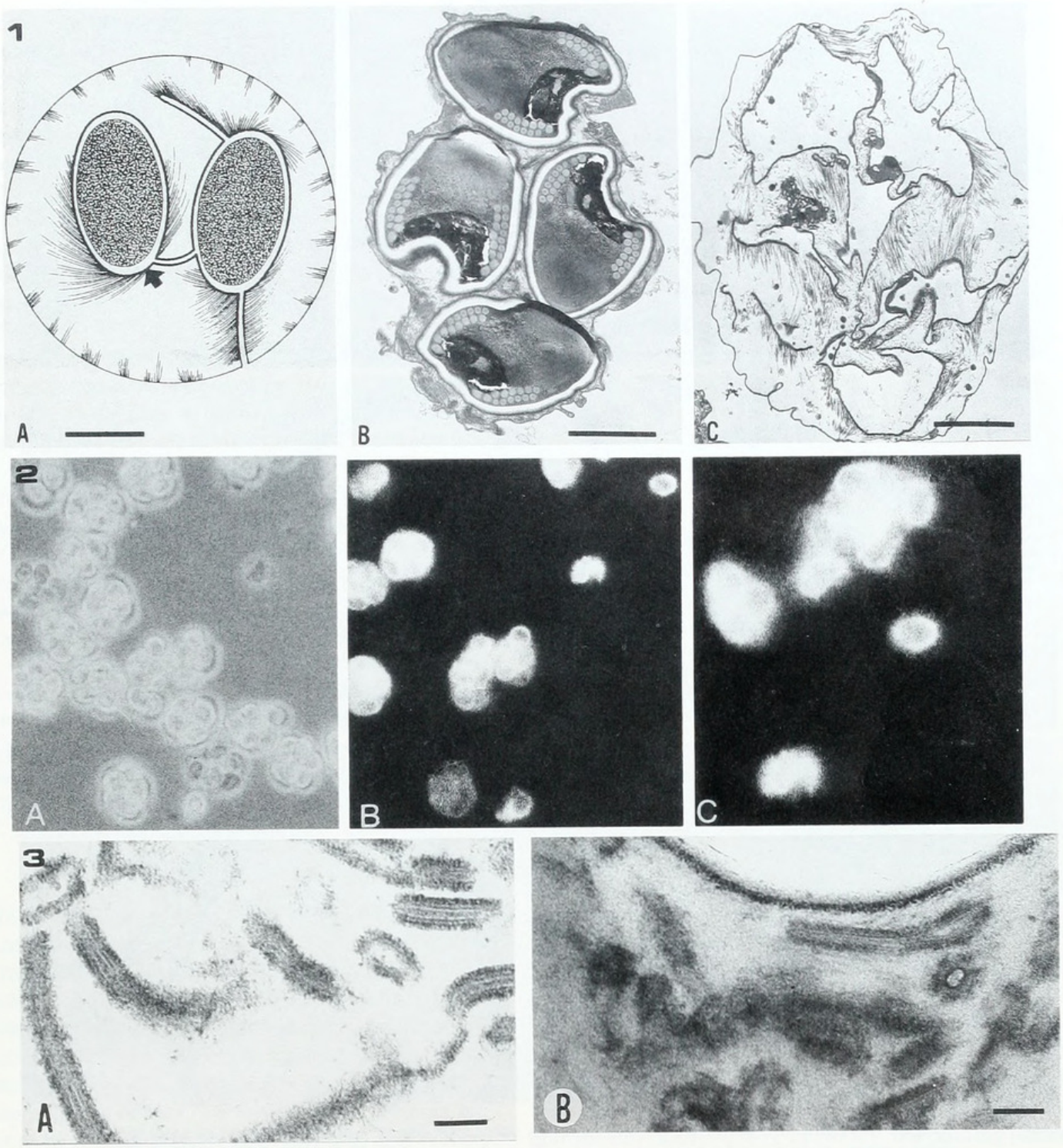

Figure 1. Low magnification images of *Thelohania* sp. spore sacs. Figure A is a diagram of a spore sac with spores. Note the arrow indicating a plaque envelope bearing cytokeratin analogues surrounding spores; plaque surrounding spores appear as half-desmosomes while plaque connecting spores appear as desmosomes. Figure B is an electron micrograph of a spore sac with spores. Figure C is an electron micrograph of a spore sac with plaque envelopes but without spores. Note the IF-bearing half-desmosome plaques that originally enveloped spores in Figure C appear like those illustrated in diagram in Figure A. Bar = 1 μm.

Figure 2. Light microscopy images of *Thelohania* sp. spore sacs. Figures A and B are phase and immunofluorescence imaging of the same field of spore sacs. About 30% of the spore sacs of *Thelohania* in Figure B were permeabilized adequately to enable anti-cytokeratin binding; all liberated spores were positive for cytokeratins. Figure C is an enlargement of Figure B; note that the fluorescence of individual cytokeratin bundles produces a fuzzy impression at the spore surface. The single spores in Figure C are 4 μm in length.

Figure 3. Electron micrographs of desmosomes within a *Thelohania* spore sac. Figure A is a lead-stained image of desmosomes. Figure B shows desmosome plaques immunoperoxidase-labeled for desmoplakin; there is no staining of the cytokeratins. Bar = 50 nm.

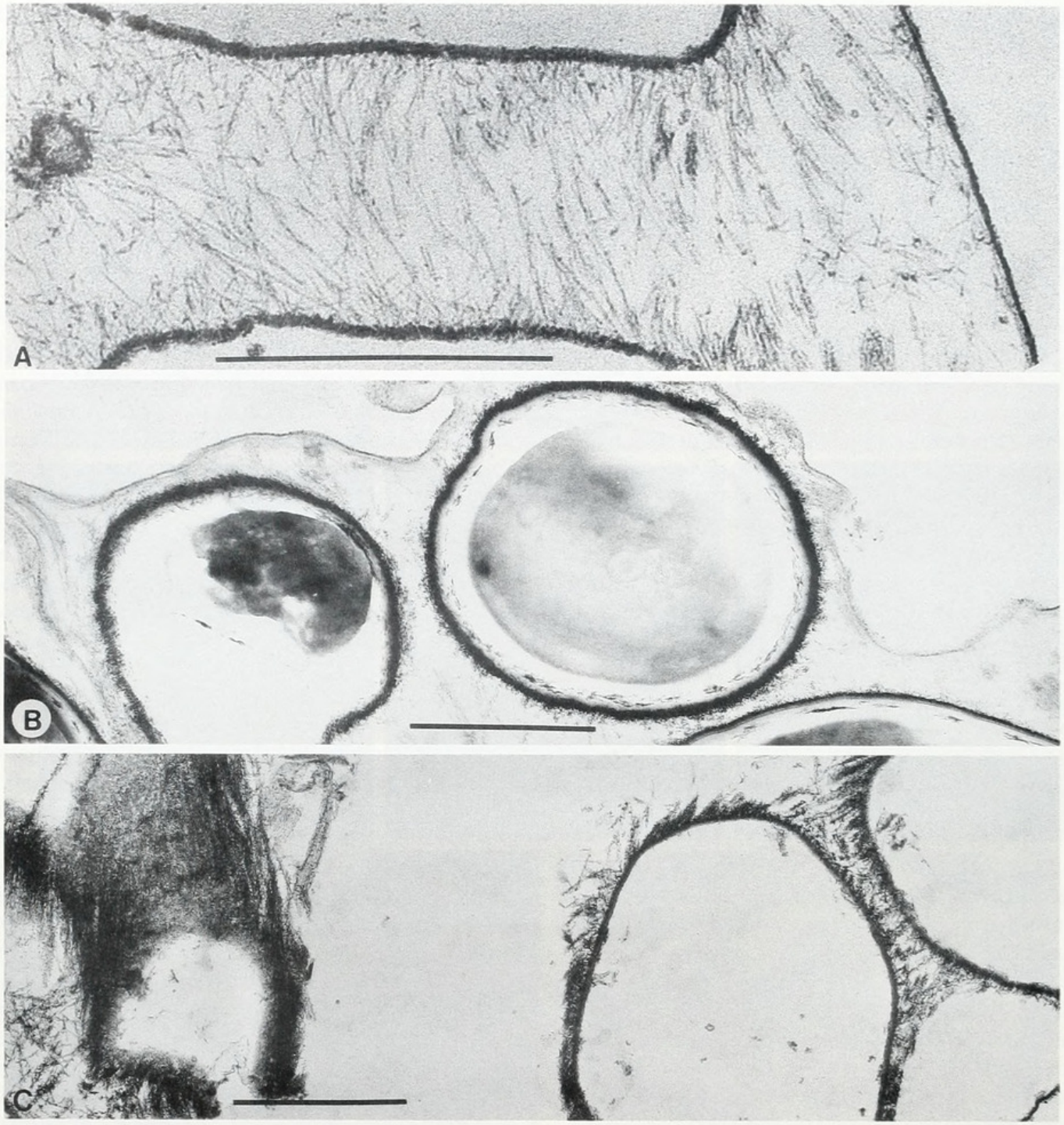

Figure 4. Electron micrographs of *Thelohania* sp. spore sacs. Figure A shows a portion of spore sac without spores. Note the half-desmosome plaques with cytokeratin analogues attached. Figure B shows the half-desmosome plaques with immunoperoxidase labeling for desmoplakin I; note that the cytokeratin analogues within the spore sac appear unstained. Figure C shows a contrasting image of half-desmosomal plaques immunoperoxidase stained for cytokeratins; note that the filaments stand out much more clearly than observed in Figure B. Bar = 0.5 μm.

for cytokeratins or for the desmosomal constituent, desmoplakin. Figure 2B shows fluorescent antibody activity for cytokeratins in field of spore sacs viewed in Figure 2A with phase optics. Figure 2C shows an enlargement of a few fluorescent antibody-labeled *Thelohania* sp. spores. The hairy surface of fluorescence at the spore surface is attributed to the spore-bound IFs. Both desmoplakin and cytokeratin antibody activities were confined to the *Thelohania* sp. spore sacs throughout the different stagings in microsporidian development; thus, host muscle tissue domains were negative for cytokeratin and desmoplakin analogues.

Ultrastructure of *Thelohania* sp. spore sacs showed an abundance of cytokeratin IFs attached to half-desmosome-

like plaques enveloping the spores (Fig. 4A). The half-desmosomal plaques that envelope each spore join to form desmosome-like attachments between the spores (Fig. 1A, Fig. 3A). Immunostaining with peroxidase conjugate directed to desmosomal indicator, desmoplakin 1, yielded peroxidase staining on both the desmosome-like structures (Fig. 3B) and the half-desmosomal analogues surrounding the spores within the *Thelohania* sp. spore sacs (Fig. 4B). Immuno-localization of antibody-peroxidase for cytokeratin analogues revealed obvious staining for bundles of IFs bound to the plaques enveloping the microsporidian spores (Fig. 4C).

For further analyses of the number of cytokeratin analogues reactive to keratin antibodies, proteins recovered from purified *Thelohania* sp. spore sacs were subjected to gel electrophoresis and immunoblotting. Monoclonal antibodies AE1 and AE3, K8.13, and Lu5 reacted to epitopes common to cytokeratin analogues from *Thelohania* sp. spore sacs. Immunoblots show response bands near positions 50, 54, and 56 kDa (Fig. 5A). Monoclonal antibody K8.12 reacted to two bands, indicating the presence of cytokeratin analogues 13 and 16 (51 and 48 kDa). For identifying desmosomal proteins, monoclonal antibodies DP2.15 and DP2.17 were used. These antibodies were responsive to a single 240 kDa band corresponding to desmoplakin 1 in *Thelohania* sp. spore sacs (Fig. 5B). In the controls, DP2.15 antibody responded only to the 240 kDa desmoplakin band from bird (turkey) wing tegument (Fig. 5C).

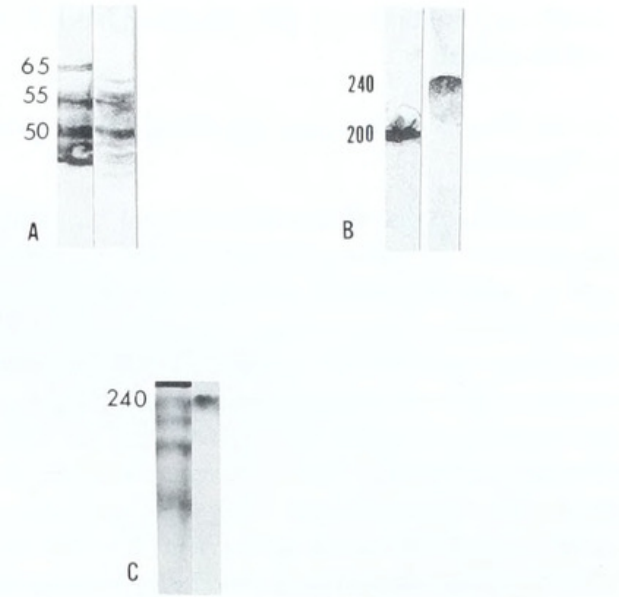

Figure 5. Immunoblot analysis of *Thelohania* sp. spore sac proteins. (A) Lane one shows standard cytokeratins resolved (from human epidermis) into bands in the 65, 58, 56 and 50 kDa range. Lane 2 shows distinct bands of 56, 54, 52, 50 and 48 kDa for cytokeratins from *Thelohania* spore sacs. (B) Lane 2 shows a 240 kDa response band for desmoplakin I; lane 1 shows the molecular weight marker myosin (200 kDa). (C) Control showing desmoplakin I. Lane 1 shows stained proteins from turkey epidermis. Lane 2 shows an immunoblot of lane 1 with a response band to desmoplakin I.

Discussion

The results of this study indicate that cytokeratin IFs and desmosomal proteins appear to be expressed within the microsporidian spore sac stages found within the skeletal muscle of the crustacean, *Callinectes sapidus*. Monoclonal antibody labeling, applied to immunofluorescence and immuno-electron microscopy, indicate an abundance of cytokeratin IFs and desmoplakin analogues within *Thelohania* sp. spore sacs. Curiously, these proteins are reported to be absent in arthropods (Bartnik and Weber, 1989). Immunolabeling data, supported by gel electrophoretic and immunoblot analyses, indicate that the cytokeratin and desmoplakin analogues recovered from the microsporidian spore sacs were immunologically responsive to antibodies prepared against bovine cytoskeletal counterparts. This is surprising because cytokeratins have diversified rather significantly among vertebrates. Antibodies directed to bovine cytokeratins would not be expected to respond to cytokeratin analogues from a lower eukaryote (Fuchs and Marchuk, 1983). Additionally, it was unexpected to find cytokeratin IFs in blue crab skeletal muscle because neither skeletal muscle nor arthropods appear to express cytokeratins. However, the cytokeratins and desmoplakin analogues were confined to the microsporidian spore sac domains within the skeletal muscle.

Two major lines of evidence indicate that the spore sac structure represents a stage in microsporidian life cycles. First, the majority of microsporidian species have spore sacs as a stage in their life cycle (Canning *et al.*, 1982; Becnel *et al.*, 1986). Second, during microsporidian spore sac development, the spores differentiate internally within a progenitor cell in which the extrasporular cytoplasmic domain becomes the spore sac (Overstreet and Weidner, 1974).

The binding of cytokeratin analogues to desmoplakin-bearing plaques in *Thelohania* sp. spore sacs resembles the cytokeratin IF attachments in vertebrate tegumental epitheilium (Kelly, 1966). However, the binding patterns differ because cytokeratin in epithelial cells binds only to membrane after it is stabilized by attachments to adjoining membrane from another cell. In *Thelohania* sp., however, the cytokeratin and desmoplakin plaque binding is primarily to that membrane which is attached to spore surfaces. Thus, cytokeratin and plaque analogues in *Thelohania* spore sacs bind to membrane that has firm attachments to spore surfaces.

The identification of cytokeratin polypeptides in *Thelohania* sp. spore sacs is very preliminary; nevertheless, cytokeratin 13 and 16 appear to be present because 51 and 48 kDa proteins respond to monoclonal antibody K8.12. Also, there is some evidence of cytokeratin 8 (des-

mosomal cytokeratin), a protein which has a 52 kDa molecular weight.

The origins of desmoplakin-cytokeratin IF expression in Thelohania *sp.*

It is unlikely that IF and desmosomal cytoskeletal genes are native to the microsporidian species in general because only a small percentage of the microsporidians express these proteins. Furthermore, it is unlikely that the microsporidians acquired the capabilities for expressing these proteins from arthropod hosts because these animals do not appear to express the cytokeratins or desmosomes (Bartnik and Weber, 1989). However, it would seem more likely that *Thelohania* sp. may have acquired the cytokeratin and desmosomal genes from a vertebrate source. This is within the realm of possibility because nearly all microsporidians begin growth in epithelial cell lines; and, nearly 100 species of microsporidians have been reported parasitizing aquatic vertebrate animals (Canning and Lom, 1986).

Acknowledgments

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