Immunocytochemical Localization of Rab 5 In Freshwater Sponge Cells Containing Algal Endosymbionts

Bethany Wallace

Carroll College, Helena, MT

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Immunocytochemical Localization of Rab 5
In Freshwater Sponge Cells
Containing Algal Endosymbionts

Submitted in partial fulfillment of the
requirements for graduation with honors to the Department of
Natural Sciences at Carroll College, Helena, Montana

Bethany L. Wallace

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This thesis for honors recognition has been approved for the Department of Natural Sciences by:

John S. Addis, Ph.D., Director

Gerald F. Shields, Ph.D., Reader

Joan Stottlemyer, Reader
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ABSTRACT

The freshwater sponge, *Ephydatia muelleri*, harbors symbiotic algae within its cells. These algae are taken up by phagocytosis, but they fail to be broken down. One explanation for the lack of degradation is that their digestion is prevented at an unknown point along the phagocytic pathway. In an attempt to determine if the vacuoles containing algal endosymbionts had characteristics associated with early endosomes, I used immunocytochemistry to localize Rab 5 in sponge cells. Rab 5 is a protein that binds to membranes and functions in docking and fusion events early in the endocytic and phagocytic pathways. Immunoblots using antibody against human Rab 5 identified three proteins, with molecular weights of 26,000, 31,000, and 46,000, in homogenates of sponge tissue. The first has a molecular weight close to mammalian Rab 5. Immunocytochemical localization revealed deposits of reaction product on the membranes of the perialgal vacuoles and in the cytosol. These results indicate that Rab 5 could be bound to the surface of the vacuoles and that vacuoles housing endosymbiotic algae could be blocked at an early stage in the phagocytic pathway.
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INTRODUCTION

The alga, Chlorella spp., enters into an endosymbiotic relationship with a number of freshwater invertebrates including the sponge, Ephydatia muelleri (Williamson, 1979). The sustainability of a relationship between Chlorella and the archaeocyte cells of the sponge is well documented (Williamson, 1979; Saller, 1991; Sand-Jensen and Pedersen, 1994). These intracellular symbiotic algae enter the degradative pathway of the archaeocyte via phagocytosis, as food particles do, but there is only limited digestion of these cells by the sponge (Saller, 1991). One possibility to explain the low rate of algal degradation is that the pathway is blocked at a point before the algae encounter digestive enzymes. This pathway normally proceeds as the phagosome matures in stages to the phagolysosome. In the lysosome, degradative enzymes break down the particles. However, these algal cells avoid digestion and maintain residence in a vesicle of the sponge (Williamson, 1979). It is unclear at what point this maturational block is reached and by what means it occurs.

The large family of Rab proteins, including Rab 5, is a subset of the low molecular weight GTPases and is responsible for directing vesicles and docking them with their targets (Waters and Pfeffer, 1999; Novick and Zerial, 1997). Different cellular compartments are characterized by different Rab proteins. Studies show that pathogenic mycobacteria survive in vacuoles carrying the early endosomal marker Rab 5, which suggests that they do not reach the level of late endosome or lysosome (Pieters, 2001).

I hypothesized that the block in the degradative pathway in E. muelleri occurs at the level of the early phagosome, as in the mycobacteria, and that the early endosomal marker Rab 5 will be found on the surface of the Chlorella-containing vacuoles. The first goal of this project was to determine if commercially available Rab 5 polyclonal antibody raised against human Rab 5 recognizes Rab 5 in the sponge via immunoblot analysis. Secondly, I attempted to localize Rab 5 within the archaeocyte, specifically, to the
Chlorella containing vacuoles. Localization of Rab 5 to the membrane of the perialgal compartment would indicate a maturational block at the early endosome-like stage.
LITERATURE REVIEW

A symbiotic relationship involves the close relationship of two organisms of different species. *Chlorella* has such a relationship with several species of freshwater sponge including *E. muelleri*. This relationship benefits both organisms. The sponge receives the products of photosynthesis from the algae, and algae are provided with a stable environment in which to live (Saller, 1991).

An important and much studied relationship is that initiated by pathogenic mycobacteria. *Mycobacterium tuberculosis* alters the maturation of the phagosome to prevent fusion with the lysosome thereby residing in an endosome-like compartment, not a lysosome-like one (Allen and Aderem, 1996). While the consequences of the internalization of *Chlorella* and mycobacteria are different, there may be similarities in the mechanism by which they survive inside the host. Rab 5 has been found on the surface of the vacuoles containing mycobacteria, giving reason to suspect that it is also present on perialgal vacuoles in *E. muelleri* (Pieters, 2001).

In the case of *E. muelleri*, as well as that of parasitic mycobacteria, the relationship between host-cell and the bacteria or algal cell is initiated by uptake by phagocytosis. Phagocytosis, in simple terms, is the internalization of large particles by a cell. The purpose of such events can be to obtain food, recycle cellular debris, or destroy invading pathogens such as bacteria. Phagocytosis is like receptor-mediated endocytosis in that they both rely on signals from the targets interacting with membrane receptors (Hahn-Keser and Stockem, 1997). These receptors then initiate the internalization of the particles by activating proteins (Allen and Aderem, 1996). In the case of phagocytosis, actin polymerization occurs and the phagocytic cup moves outward to surround the particle. However, endocytosis relies on clatherin-coated pits which pull the particle into the cell and acts independently of the actin-based cytoskeleton (Allen and Aderem, 1996).
Endocytosis and phagocytosis also differ in the pathway taken after internalization (Rupper and Cardelli, 2001). While the pathway from a phagosome to a mature phagolysosome is not exactly the same as the pathway from an early endosome to a lysosome, there is evidence that phagosomes have limited, incomplete fusion and fission interactions with the endocytic pathway in which phagosomes acquire transmembrane proteins found in the endosomal-lysosomal pathway (Allen and Aderem, 1996). This has been termed the “kiss and run” mechanism (Hahn-Kesser and Stockem, 1998). Rab 5 is among the proteins obtained by phagosomes during maturation (Aderem and Underhill, 1999).

Rab proteins are a family of low molecular weight GTPases associated with endosomal trafficking in the cell and directing fusion events (Chavrier and Goud, 1999). Rab 5 is characteristic of the early endosome and is needed for homotypic fusion between early endosomes and probably for fusion between maturing phagosomes and early endosomes (Aderem and Underhill, 1999). Rab 5 is present on the plasma membrane and on the early endosome in the active GTP form. Once Rab 5 has performed its duties in endosome fusion, it is released into the cytosol and is found there in its inactive GDP form (Waters and Pfeffer, 1999; Novick and Zerial, 1997).
MATERIALS AND METHODS

Sponge Collection and Maintenance

*E. muelleri* was collected from Salmon Lake, Montana and maintained in an aerated, water jacketed aquarium up to 7 d. The aquarium was illuminated by a Gro-light (Sylvania) for 14 h per day from a height of 50 cm.

Dissociation of Cells

Prior to experimentation, cells were dissociated and spicules were filtered out. Approximately 3-4 g of sponge tissue (~2 x 2 x 0.5 cm) were covered with 6 ml calcium/magnesium free medium (CMF, 6 mM Tris + 34 mM sodium chloride + 1.34 mM potassium chloride + 1.38 mM glucose + 0.07 mM K2HPO4 + 1.07 mM NaHCO3 + 0.25 mM EDTA, pH 7.6) and minced finely with a clean razor blade. The slurry was then transferred to a tube, capped, and allowed to settle for 5 min. The solution was vortexed at a medium setting of a vortex mixer 5 times, 5 s each, then poured into a syringe attached to a cartridge filter containing 44 µm Nitex mesh and collected in a centrifuge tube. This solution was centrifuged at 1000x g for 15 s. The CMF was pipetted off and cells were resuspended in low salt phosphate buffered saline (PBS-LS, 50 mM sodium chloride + 5 mM sodium phosphate buffer, pH 7.2).

Immunoblot Analysis

Approximately 4 g of fresh sponge tissue were resuspended in 500 µL PBS-LS and homogenized by hand in a glass homogenizer with a teflon pestle using 5 up/down strokes. The homogenate was stored on ice until use. Protein concentration was determined using the Bradford assay with bovine serum albumin (BSA) standards. Polyacrylamide gels (12%) were made and loaded based on recommendations for the Mini-Protean II electrophoresis unit (Bio-Rad). The reference lane was prepared with 1.5 µL (total 3 µg per protein standard) low molecular weight standards (Bio-Rad). Two experimental samples were prepared using the homogenate such that they contained 75...
and 150 μg of total protein; and a fourth lane included 10 μg BSA. Sample buffer containing 2% (w/v) bromophenol blue was used rather than the recommended 1% to better locate the dye front during the electrophoretic run. A volume of 15μL of each sample was loaded onto the gel. Samples were electrophoresed at 150V through the stacking gel and 200V through the separating gel. The diffusion blot was set up by constructing a sandwich, from the outside to the center containing a plastic framework, a layer of Scotchbrite® scouring pads, four layers of filter paper, and a layer of nitrocellulose on each side of the trimmed gel. Each layer was individually soaked in transfer buffer prior to addition of the gel. The sandwich was held together with rubber bands and submerged in 3 L of transfer buffer, composed of 25 mM Tris (3.0 g/L), 192 mM glycine (14.42 g/L), and methanol (200 mL/L) in water, overnight.

One nitrocellulose membrane was stained for protein. The other was reserved for immunodetection of Rab 5. To stain for protein, the membrane was submerged in 0.1% (w/v) amido black in 45% methanol, 10% acetic acid for approximately 70 s, or until dark bands appeared against a lighter background. The membrane was then destained in 90% methanol, 2% acetic acid for about 2 min. The membrane was placed in water until it sank and was then blotted dry with filter paper.

The second membrane was placed in a plastic bag and washed 3x with PBS (150 mM sodium chloride + 5 mM sodium phosphate buffered saline, pH 7.4), 3 min each. The PBS was poured off and replaced with 10 mL 3% BSA/PBS and rocked at 4° C overnight. The membrane was then placed in a clean bag with 2 mL of a 1:200 dilution of the polyclonal Rab 5 antibody (Stressgen Biotechnologies) in 3% BSA/PBS for 2.5 h. It was then washed 4 times, 5 min each, with PBS in a new bag. Then the secondary antibody, a 1:3000 dilution of anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) in BSA/PBS was added for a 45 min incubation followed by one 5 min rinse in PBS and two 5 min rinses in water. Finally, the membrane was incubated in BCIP/NBT color
reagent (Sigma) until bands became visible (approximately 5 min). The membrane was rinsed well with water and blotted dry on filter paper.

**Immunocytochemistry**

Dissociated cells were allowed to settle to the bottom of the tube in CMF so that most of the CMF could be pipetted off; the remaining cells were transferred to a microcentrifuge tube. The cells were centrifuged at 1000x g for 15 s. The remainder of the CMF was removed, and the pellet was just covered in PBS-LS and resuspended. Then 2 volumes of O.C.T. compound (Tissue-Tek) were mixed into the suspension. The mixture was frozen in an ethanol and dry ice bath. The frozen plug was removed from the tube and mounted on a block for sectioning in the cryostat. Sections were affixed to gelatin coated slides by allowing them to melt by placing a finger on the bottom of the slide.

The slides were then treated as described by Chavrier et al. (1990). Cells were first permeabilized in a solution containing 200 mM PIPES-KOH (pH 6.8), 20mM EGTA, 4 mM MgCl₂, and 2% saponin for 10 min. Cells were then fixed for 15 min in 3% formaldehyde. Two rinses (2 min each) in PBS/0.5% saponin and 3 rinses in PBS/0.5% saponin +10% goat serum followed. The cells were incubated with the primary Rab 5 antibody for a period of 3 h in a humidified chamber at a dilution of 1:50. Controls received an equivalent dilution of non-immune rabbit serum. Slides were then rinsed 3X in PBS/saponin+goat serum, 5 min each. The secondary antibody incubation was a 1:100 dilution of anti-rabbit IgG conjugated to alkaline phosphatase for 45 min. Cells were rinsed 3 times in PBS and incubated in Fast Red/Naphthol AS-MX (Sigma) for 5 min. The slides were mounted in glycerol, observed by bright field microscopy, and photographed.
RESULTS AND DISCUSSION

The results of the immunoblot indicate bands representing three different proteins in the sponge with molecular weights of 46,000, 31,000 and 26,000 (Figure 1). The intensities of the three bands are approximately equal. The molecular weight 26,000 band recognized by the antibody has a molecular weight corresponding to mammalian Rab 5 and thus could represent the sponge Rab 5. The 31,000 and 46,000 proteins are unknown but have structural affinities with Rab 5. Stressgen, the company from which the antibody was obtained, reports that there may be an additional band at 45,000 which is likely the band observed at approximately 46,000.

Immunocytochemical localization of Rab 5 is shown in Figure 2. The micrographs each show a single archaeocyte cell from samples of E. muelleri containing perialgal vacuoles. Reaction product, indicating the presence of the sponge proteins recognized by the human Rab 5 antibody, is red. Light red is seen in the cytosol and darker red is present on the membranes of the perialgal vacuoles (yellow-green disks) in the micrographs (Figure 2 a-c). Dark areas of staining indicating localized protein, are present in experimentals but are absent in controls. Background staining of the experimental cells is much darker than that of controls (Figure 2 d).

In the immunocytochemical localization, there appears to be a build-up of reaction product localized to the perialgal vacuoles. Rab 5, therefore, could be present and these compartments could be early endosome-like. There also appears to be localization to the plasma membrane and the cytosol, which is compatible with the location of Rab 5 in mammalian cells (Novick and Zerial, 1997; Segev, 2001). More definitive results could be obtained with an antibody that recognizes only one protein to determine if Rab 5 is present.

Some Rab 5 is cytosolic, which might account for heavy background staining (Novick and Zerial, 1997). This staining might be reduced by using a lower
concentration of the primary antibody while still providing an adequate buildup of color
to localize the sponge proteins on membrane surfaces.

In the future it might also be interesting to localize Rab 7, a late endosomal
marker, or another early endosomal marker, in these cells as further conformation of the
nature of these perialgal vacuoles.
Figure 1. Immunodetection of Rab 5 protein in *E. muelleri* on western blot. Bands have molecular weights of (from top to bottom) 46,000, 31,000 and 26,000.
Figure 2. Immunocytochemical localization of Rab 5 in sponge archaeocytes. (a), (b), and (c) are experimental cells; (d) is a control. Arrowheads indicate heavy deposits of reaction product on perialgal vacuole membranes.
LITERATURE CITED


