

Immunocytochemical Localization of Rab5 in Perialgal Vacuoles of a Freshwater Sponge

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Abstract

Chlorella sp. is a green alga that exists in endosymbiotic relationships with a number of freshwater invertebrates including the sponge, *Ephydatia muelleri*. Algae enter sponge cells through phagocytosis, but unlike most cells taken up by this process, the algal cells are not degraded. The mechanism by which *Chlorella* escapes degradation is currently unknown. One hypothesis is that the vacuoles containing algal cells do not progress along the degradative pathway but instead are maintained in an immature, pre-lysosomal state, displaying the early endosomal marker Rab5. In order to test this hypothesis, I localized Rab5 in sponge cells using immunocytochemistry. Two antibodies against human Rab5 were first shown by Western blotting to bind to a sponge protein having a molecular weight similar to that of mammalian Rab5. Both antibodies also recognized some higher molecular weight proteins. From these results, I concluded that there was sufficient cross reactivity to use the antibodies for immunocytochemistry. I was unable to localize Rab5 in cells using one of the antibodies; however, with the other, I detected antibody bound to algae-containing vacuoles. This latter result suggests that vacuoles containing symbiotic algae may express Rab5 and that maturation of the vacuoles may be hindered.

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Introduction

The alga, *Chlorella sp.*, enters the sponge, *Ephydatia muelleri*, through the process of phagocytosis and forms an endosymbiotic relationship with the sponge (Penney and Racek, 1968). Particles obtained through the process of phagocytosis normally are digested; however, in the *Chlorella/Ephydatia* symbiosis, this process is interrupted, allowing the algal cells to survive. The vacuoles containing the symbiotic *Chlorella* are not markedly acidic ($> \text{pH } 5.7$) (Rands et al. 1993); this suggests that the vacuole does not reach a lysosomal stage while the *Chlorella* is still alive.

Investigations of symbiotic relationships indicate that microbes can escape digestion by interfering with phagosomal maturation; however, the mechanism used is still not completely understood (Pieters 2001). Most research has focused on bacteria that are able to escape degradation; most of these bacteria are mycobacteria. Mycobacterial phagosomal compartments (MPC) do not appear to fuse with lysosomes while the bacteria are still living (Meresse et al. 1999). The fusion of an MPC with a lysosome would cause the vacuole to become acidic and surface marker proteins to change (Meresse et al. 1999). Localization of surface marker proteins indicates that these vacuoles are not acidified because they never reach the late endosome or lysosome stage (Meresse et al. 1999).

The phagocytic pathway is regulated by a family of monomeric GTPase proteins called Rabs (Novick and Zerial 1997). Rab5 is the prominent early endosome marker (Novick and Zerial 1997). This protein guides the vacuole from early to late endosome stages. Rab5 recruits and binds EEA1 (early endosomal antigen 1), a Rab5 effector

protein, facilitating the transformation from early to late endosome vacuoles (Fratti et al. 2001).

I hypothesized that the alga, *Chlorella sp.*, escapes degradation within *E. muelleri* by remaining contained within immature, prelysosomal vacuoles. This hypothesis was tested by localizing Rab5 in sponge cells using immunocytochemistry. Western blots were first conducted to determine the amount of cross reactivity between anti-human Rab5 antibodies and sponge Rab5. Two antibodies were chosen as candidates for immunocytochemistry based on the results of the Western blots.

Literature Review

Endocytosis/Phagocytosis – An overview

Phagocytosis involves the ingestion of extracellular material by a cell and ordinarily leads to the degradation of that material. According to Vieira et al. (2002), phagocytosis begins with an interaction of surface receptors with a cognate ligand (such as lipoprotein of a bacterial cell wall). Receptor stimulation leads to the internalization of the particle through the movement of the cytoskeleton, which results in the formation of a phagocytic vacuole or phagosome. The phagosome initially formed has constituents of the extracellular space and a membrane similar to that of the plasma membrane. The vacuole proceeds along an endocytic pathway that is chaperoned by a series of small molecules. Initially the vacuole is termed an early endosome and is marked by the acquisition of Rab5 and EEA1. Rab5 is the main identifying protein of early endosomes, and these endosomes have been found to have a pH ~ 6.0. Progression to the late endosome stage is signaled by the accumulation of Rab7 and Rab9 and the loss of Rab5

from the vacuole membrane. Late endosomes are more acidic than early endosomes (pH ~ 5.5) and tend to be enriched with hydrolytic enzymes. The final step in the endocytic pathway is the maturation of the late endosome into a lysosome. Lysosomes tend to be more acidic than late endosomes (pH ~ 4.5) and express lysosomal-associated membrane proteins (LAMPs).

Rab 5 in the Endocytic Pathway

Rab5 is the prominent early endosome marker (Novick and Zerial 1997). This protein guides the vacuole from early to late endosome stages. Rab5 recruits and binds EEA1, a Rab5 effector protein, to facilitate the transformation from early to late endosome vacuoles (Fratti et al. 2001).

According to Meresse et al. (1999), Rab5 has been implicated in the transport of mycobacterial phagosomal compartments (MPC), compartments within a cell that contain mycobacteria. The cell acquires the mycobacteria through the process of phagocytosis. MPCs do not appear to fuse with lysosomes while the bacteria are still living. The fusion of an MPC with a lysosome would cause the vacuole to acidify and surface marker proteins to change. Localization of surface marker proteins indicates that these vacuoles are not acidified because they never reach the late endosome or lysosome stage.

Fratti et al. (2001) studied *Mycobacterium tuberculosis* to determine how the mycobacterium was able to avoid degradation within a phagocyte. They observed that the maturation block occurred close to the Rab5 end of the Rab5-to-Rab7 transition and EEA1's recruitment was found to be the critical component of this process. When EEA1 did not accumulate the vacuole did not progress to the late endosome stage and Rab7 was not acquired (Fratti et al. 2001).

According to Duclos et al. (2000), in the protozoan parasite, *Leishmania donovani*, Rab5 is the protein that influences the progression of phagocytosis and vacuole fusion with endosomes. The importance of Rab5 was studied through the use of mutant Rab5 proteins. Rab5 (Q79L) is a mutant Rab5 that has inhibited GTPase activity. As a result, membranes expressing Rab5 (Q79L) caused the formation of extremely large phagosomes containing many parasites. The usual division of *L. donovani* is accompanied by the fission of the phagosome, which results in the separation of the daughter cells into separate endosomes; thus the parasites do not become concentrated within a vacuole. Rab5 was identified as an important molecule in the process of fusion between endosomes and phagosomes. When Rab5 (Q79L) was present on the phagosomes, the fusion time was extended and more material was introduced into the phagosome causing it to swell.

Chlorella/Freshwater Sponge Symbiosis

The alga, *Chlorella sp.*, is associated in symbiotic relationships with many invertebrates, but it is not completely dependent upon its host and can live on its own (Saller 1991). According to Saller (1991), *Chlorella sp.* enters the sponge, *Spongilla lacustris*, through the process of phagocytosis and forms a mutualistic endosymbiotic relationship. *Chlorella* receives protection and the sponge receives nutrients that have been produced through photosynthesis by the algae.

In the *Chlorella/Ephydatia* symbiosis the phagocytic pathway is interrupted in order to maintain the integrity of the algal cells. Evidence for the interruption of this process is that vacuoles containing the symbiotic *Chlorella* have not been found to be markedly acidic (> pH 5.7) (Rands et al. 1993). Since an acidic vacuole is not present, the

retention process may be explained by the vacuole's not reaching the lysosome stage or by its removal from the pathway.

Materials and Methods

Collection and Storage of Living Sponge

Samples of *Ephydatia muelleri* were collected from Salmon Lake (47°06'N/113°24'W) and Coopers Lake (47°05'N/112°55'W) Montana. The sponge was removed from the substratum (usually rocks or submerged wood) and placed into containers filled with water from the collection site. The sponge was maintained for 24 hr in an aquarium that had been filled with filtered water from the collection site in order to allow passage of undigested food particles through the phagocytic pathway. The water was aerated and kept cold with tap water. The specimens were illuminated by a single 24 inch lamp (Gro-Light, Slovenia) positioned approximately 30 cm above the aquarium and programmed on a 14 hr light/10 hr dark cycle.

Cell Dissociation

For this and subsequent procedures, I followed the methods described by Wallace (2001) with a few modifications. Approximately 3 g of sponge were mixed with 6 mL of Ca^{2+} and Mg^{2+} free medium (CMF) (6 mM Tris-HCl pH 7.6, 34 mM NaCl, 1.34 mM KCl, 1.38 mM glucose, 0.7 mM K_2HPO_4 , 1.07 mM NaHCO_3 , 0.25 mM EDTA, pH 7.6). The sponge was diced with a razor blade in CMF and then transferred to a 15 mL plastic culture tube. It was allowed to stand for 5 min and was vortexed 5 times for 5 sec each at setting 7 (Fisher Vortexer 2). The vortexed mixture was filtered through either a 44 μm or 125 μm Nitex mesh. The resulting filtrate was placed in centrifuge tubes and centrifuged

for 15 s at 1000xg. The supernatant was discarded and the pellet was resuspended in PBS-LS (low salt phosphate buffered saline, 50mM sodium chloride, 5mM sodium phosphate). The pellet was resuspended either in: 1) in PBS plus or minus 5mM EDTA and 0.4mM PMSF (phenyl methane sulfonyl fluoride, Sigma), homogenized, and frozen at -20°C or 2) in Poly-Freeze and frozen at -70°C .

Western Blot Staining

Sponge proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels. The gels were loaded with approximately 50 μg of protein. Protein was determined using the Bradford assay (Bio-Rad). The protein was transferred from the gels to nitrocellulose membranes (Sigma) by diffusion. The gels and membranes were immersed either in a transfer buffer (25mM Tris, 192mM glycine, 20% methanol) or a renaturing transfer buffer (10 mM NaHCO_3 , 3 mM Na_2CO_3 , pH 9.8). If the renaturing buffer was used, a renaturing wash (20% glycerol, 6mM Tris-HCl) preceded the transfer. For protein localization in the membranes, two different anti-human Rab5 antibodies were used: antiRab5 (Stressgen) and antiRab5a (Santa Cruz). The Stressgen antibody was used at a dilution of 1:200 in blotto (5% nonfat dry milk, 0.05% Tween 20/PBS) for the primary incubation. The Santa Cruz antibody was used at a dilution of 1:50. Both antibodies were incubated with the membranes at room temperature for 1 hr or overnight at 4°C . Then, the membranes were washed three times for 3 min each in 0.05% Tween 20/PBS. The secondary antibody (goat anti-rabbit IgG, alkaline phosphatase conjugate, Sigma) was incubated with the membrane at a dilution of 1:1000 for 30 min at room temperature. After the secondary antibody incubation, the membrane was washed three times for 3 min each with 0.05% Tween 20/PBS followed

by a wash for 3 min with deionized water. BCIP/NBT (Sigma) color reagent was used to localize bound antibody. It was prepared by placing half a tablet in 5 mL deionized water. The membrane was incubated for about 5 min or until bands were visible. Then the membranes were rinsed with deionized water and stored in a dark, dry place.

Immunocytochemistry

Slides were prepared by sectioning the cells frozen in the Poly-Freeze medium with a cryostat. The sections were mounted on gelatin-coated slides and kept frozen until used. The slides were brought to room temperature and treated with PBS for 5 min. Cells were permeabilized in most experiments in 50mM Pipes-KOH (pH 6.8), 5mM EGTA, 1 mM MgCl₂, 2% (w/v) saponin for 10 min. In some experiments, this solution was replaced with 0.2% Triton X-100. After permeabilization, the slides were treated with 3% formaldehyde/PBS-LS for 15 min, then 0.5% saponin in PBS twice for 5 min each. Finally, the slides were treated with 0.5% saponin/10% goat serum in PBS three times for 5 min each. Experimental slides were treated with the primary antibody and control slides were left covered by 0.5% saponin/10% goat serum in PBS. With the Stressgen antibody, the cells were incubated at a 1:50 dilution of antibody in 5% saponin/10% goat serum for 1 to 3 hr at room temperature or overnight at 4°C. With the Santa Cruz antibody, the cells were incubated under the same conditions at a 1:25 dilution in 5% saponin/10% goat serum in PBS. Primary antibody incubation was followed by three rinses of 5% saponin/10% goat serum in PBS for 5 min each. All slides were treated with the secondary antibody (goat anti-rabbit IgG, alkaline phosphatase conjugate, Sigma) (1:100) for 30 min, three rinses of PBS for 10 min each, Fast Red/Naphthol As-Mx for 5 min, and finally, three rinses for 2 min each with PBS. The slides were covered with glycerin, and

a cover slip was placed on top of the slide. The slides were viewed with a bright field microscope.

Results

Western Blots

Western Blots were conducted in order to determine whether the antibodies against human Rab5 recognized the corresponding sponge protein. The Stressgen antibody localized a protein band in the mouse homogenate and the sponge homogenate at approximately 26 kDa (Fig. 1). This is the expected size of Rab5. The Stressgen antibody also localized a protein band at about 45 kDa in the mouse homogenate. This higher band is expected as it is described by Stressgen. Other higher molecular bands were also present in the sponge lane. Although strong staining of mouse proteins corresponding to Rab5a occurred with the Santa Cruz antibody, the staining of sponge proteins was weak (Fig 2).

Immunocytochemistry

Stressgen-treated intact sponge cells. Control cells did not exhibit any staining (Fig. 3). Intact experimental cells treated with the Stressgen antibody showed cytoplasmic staining (Fig. 4). Diffuse staining was present between vacuoles, and it appeared that the surfaces of the vacuoles were heavily stained.

Stressgen-treated lysed sponge cells. Lysed cells were observed to have stained vacuole membranes indicating that Rab5 was present on these vacuoles (Figs. 5 and 6). Stained vacuole membranes were observed separating from the algal cell, but no staining was observed on the exposed algal surface (Fig 5). Staining observed around the algal

cell is due to the presence of the vacuole membrane surrounding the algal cell. Control lysed cells show no staining on vacuole or algal cells (Fig. 7).

Santa Cruz-treated intact sponge cells. Both the control and the experimental cells treated with Santa Cruz antibody showed no staining, indicating the failure of the antibody to bind to Rab5 under the conditions used (Fig. 8 and 9).

Discussion

The Stressgen antibody detected a band at the approximate size of mammalian Rab5. This result indicated that the antibody could be binding to Rab5 in the sponge. Unfortunately, several higher molecular weight bands were also present, complicating the interpretation of the immunocytochemical results.

In order to substantiate results obtained from the Stressgen antibody and to obtain further information about the form of Rab5 involved in the process, I also used anti-human Rab5a antibody from Santa Cruz. It also detected a band at about the expected weight of Rab5a. The Rab5a band was faint suggesting that the cross reactivity of anti-human Rab5a antibody was minimal. When intact cells were treated with the Santa Cruz antibody there was no observable staining.

The Stressgen antibody bound to the vacuoles surrounding algal cells. The appearance of staining around algal cells and the absence of staining on other vacuoles is consistent with the hypothesis that algae-containing vacuoles display Rab5. Staining was observed only in the cytoplasm and on algae-containing vacuoles. These data support the hypothesis that algae-containing vacuoles display Rab5 and therefore may be arrested in the early endosome stage. The Stressgen antibody did localize other bands on the

Western blot; therefore, the antibody may be localizing a protein other than Rab5 within the cell.

This research supports the hypothesis that phagocytized organisms are able to hinder progression of their vacuole in an early endosome-like state. The mechanism that organisms are using to arrest their vacuoles is still unknown. Further research into the interactions between the endocytized organism and the host cell's vacuole may lead to additional clues about how this process is occurring.



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COLLECTION

W. SOUTHWORTH

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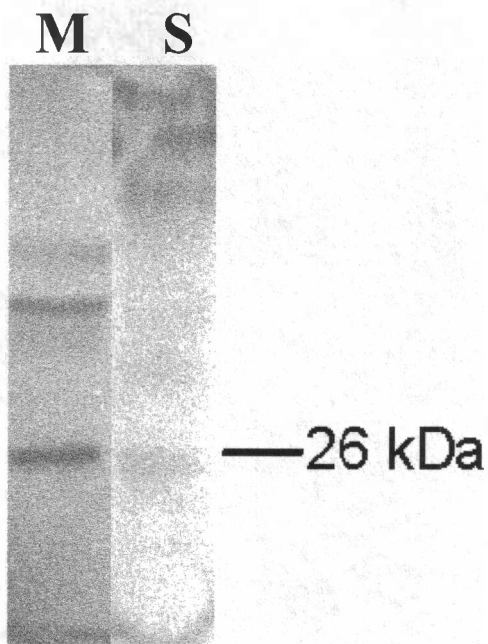


Figure 1. Western Blot treated with antibody directed against human Rab5 (Stressgen). Mouse brain control is in the left lane and sponge homogenate is in the right lane. Band indicated corresponds to the expected size of mammalian Rab5.

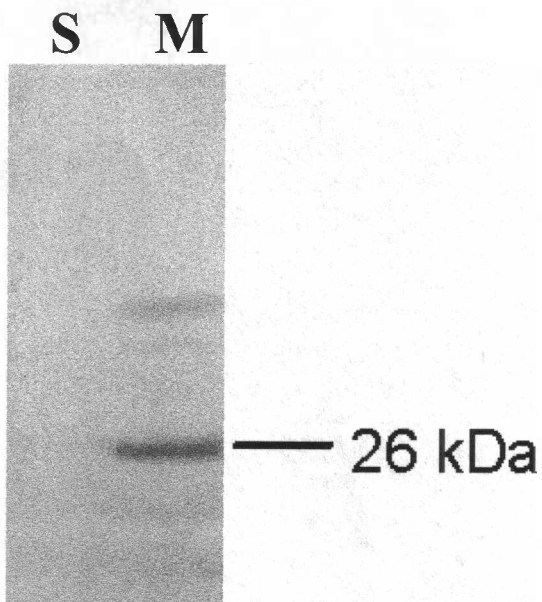


Figure 2. Western Blot treated with antibody directed against human Rab5a (Santa Cruz). Mouse brain control is in the right lane and sponge homogenate is in the left lane. Band indicated corresponds to the expected size of mammalian Rab5.

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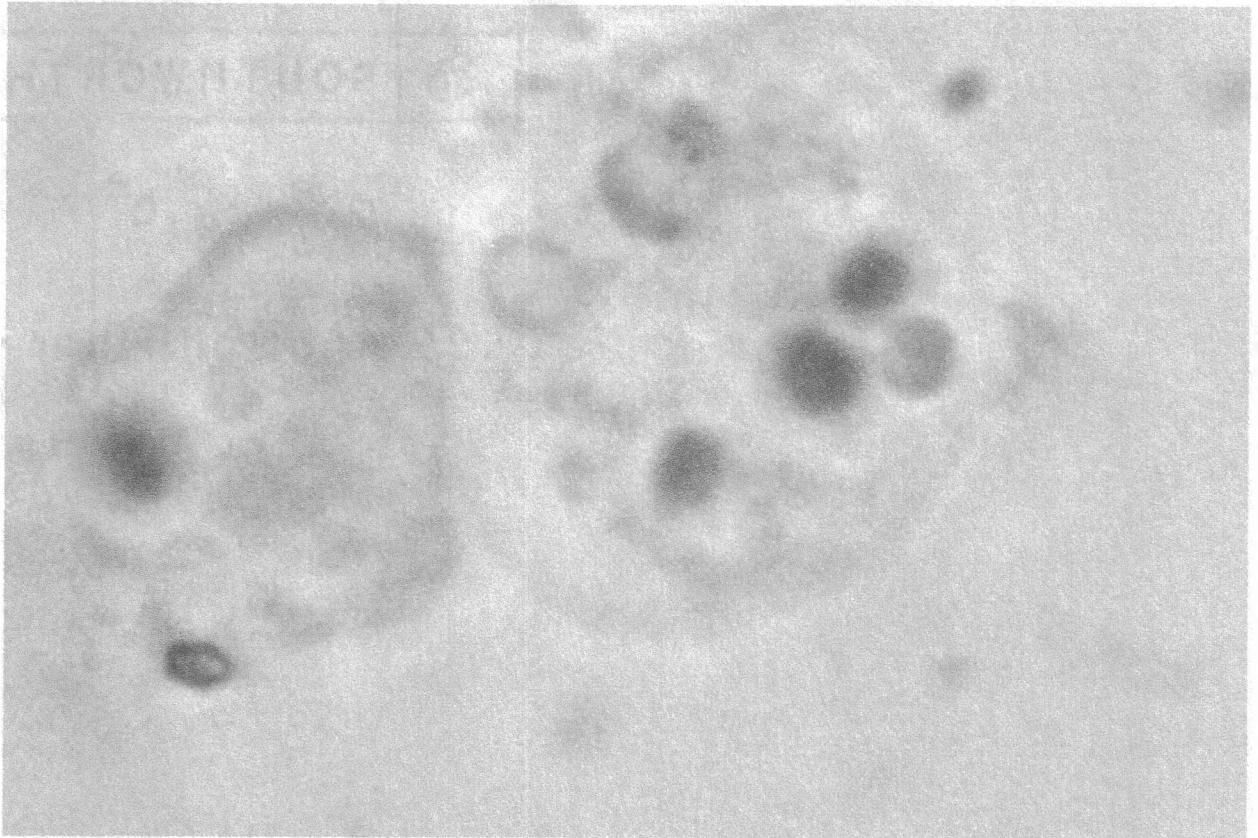


Figure 3. Intact sponge cells permeabilized and fixed (control). Note the absence of staining.

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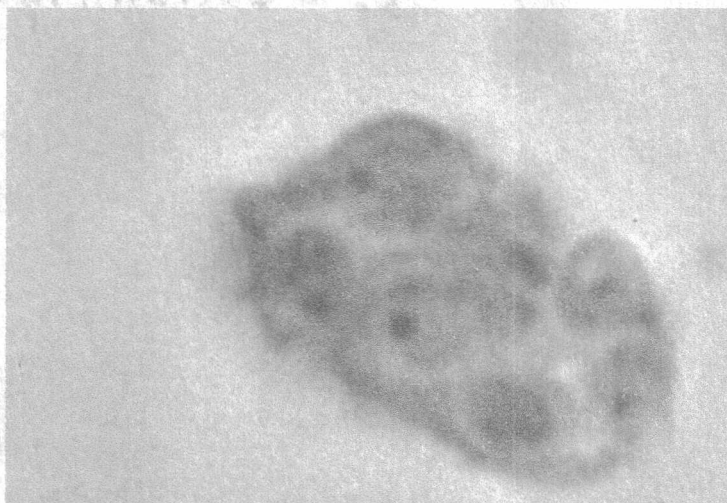


Figure 4. Intact sponge cells permeabilized , fixed and incubated with anti-human Rab5 antibody (Stressgen). Note staining in the cytosol with the heaviest staining occurring around the vacuoles.



Figure 5. Lysed sponge cells permeabilized and fixed. Notice the absence of red on the slides. Algal cells contained in vacuoles are still intact.

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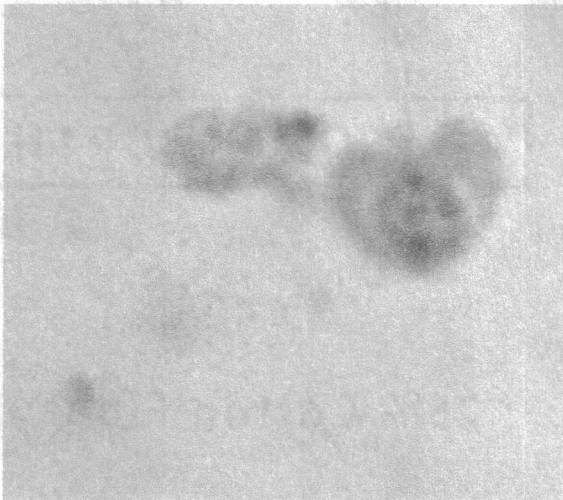


Figure 6. Lysed sponge cells permeabilized, fixed, and incubated with anti-human Rab5 antibody (Stressgen). The vacuole membrane is separating from the algal cell and is noticeably stained.

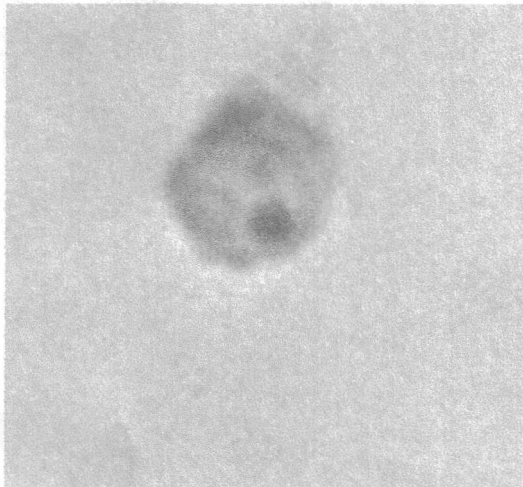


Figure 7. Lysed sponge cells permeabilized, fixed, and incubated with anti-human Rab5 antibody (Stressgen). The vacuole membrane is stained and is not part of the algal membrane because the vacuole membrane appears to have been disrupted and is separating from the algal cell.

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Collection

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GRAND

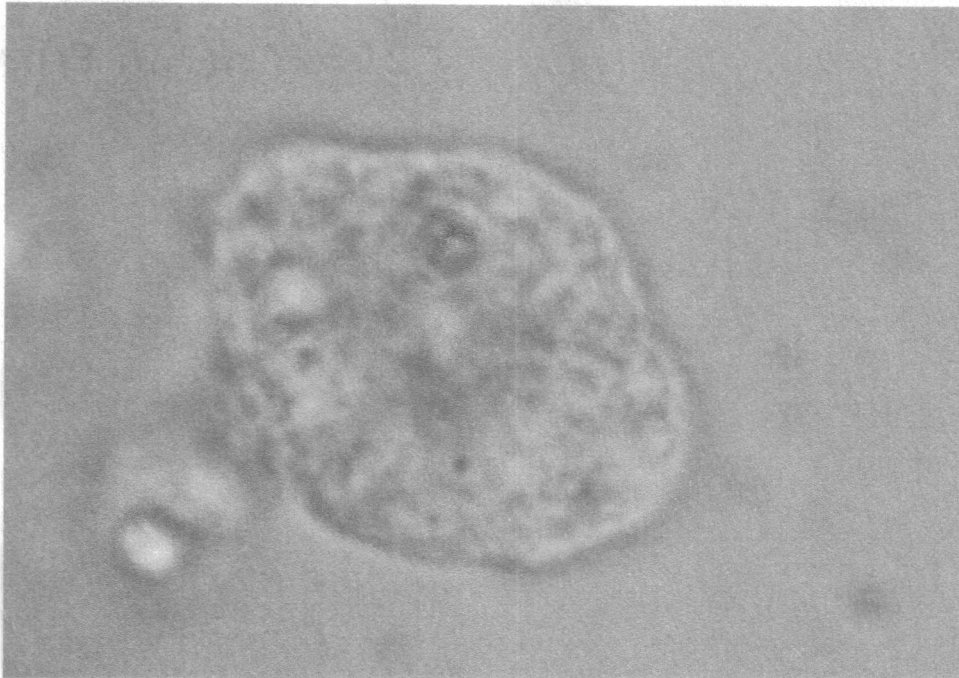


Figure 8. Intact sponge cell permeabilized and fixed.

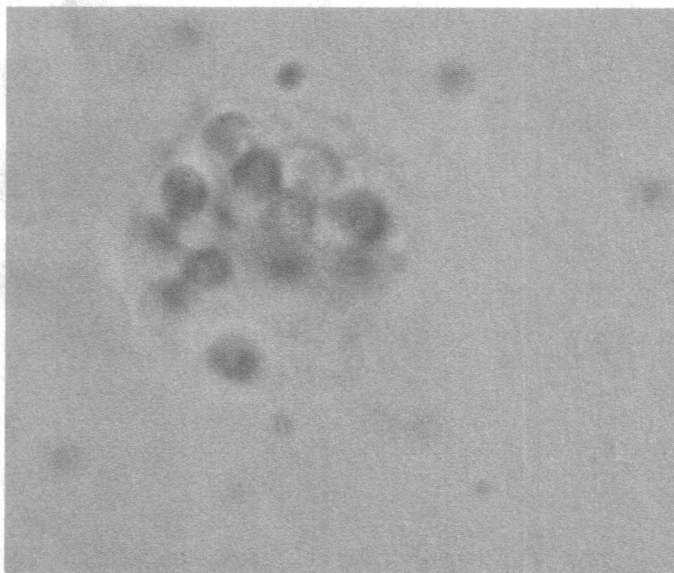


Figure 9. Intact sponge cell permeabilized, fixed and incubated with anti-human Rab5a (Santa Cruz). The antibody did not appear to associate with any protein because of the lack of red coloring.