


Localization of the Late Endosome Marker Rab7 in the Freshwater  
Sponge *Ephydatia muelleri*

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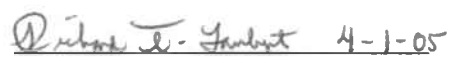
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### Abstract

The green alga, *Chlorella*, exists in a symbiotic relationship with the freshwater sponge, *Ephydatia muelleri*. *Chlorella* is taken up by phagocytosis and incorporated into phagosomes. However, the phagosomes containing the algae fail to fuse with lysosomes, resulting in a sustained symbiotic relationship. One possible explanation for the retention of algae in non-lysosomal vacuoles is that the phagosomes do not acquire the surface proteins needed for fusion with lysosomes. Among these proteins is Rab7, which has been suggested to regulate the transition from phagosome to lysosome. I hypothesized that the vacuole containing the symbiotic algae is early endosome-like and thus Rab7 is not present on the algal-containing vacuole membrane. Immunocytochemistry with antibody directed against human Rab7 was used in an attempt to localize Rab7 in the sponge cells. Western blot analysis was used to establish the specificity of the antibody. The results of both the Western blots and immunocytochemistry were inconsistent. Banding in the Western blots was faint, and the pattern of staining varied with the concentration of the primary antibody. Similarly, localization of Rab7 by immunocytochemistry was inconclusive. The inconsistency in both the Western blots and immunocytochemistry indicates that more research is needed to clarify the role Rab7 plays in establishing the symbiotic relationship between algae and sponge.

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## Introduction

The green alga, *Chlorella sp.*, exists in a symbiotic relationship with freshwater sponges, such as *Ephydatia muelleri* (Penney and Racek, 1968). *Chlorella* is engulfed by phagocytosis and incorporated into phagosomes as if it were to follow the normal digestive pathway; however, it escapes degradation, surviving inside vacuoles (Saller, 1991; Williamson, 1979). The nature of these vacuoles and the mechanism by which algal cells avoid degradation are currently unknown (Williamson, 1979). Rands et al. (1993) found that vacuoles containing either *Chlorella* or *Symbiodinium* algae were not acidic. This observation indicates that the algae are not surviving inside the lysosomes, an acidic degradative organelle. Studies done with mycobacteria suggest that phagosomes that are not acidified do not fuse with lysosomes (Barker et al., 1997). Research with both parasitic and symbiotic relationships suggests that protein markers of the endosome, such as those of the Rab family, could be involved in avoiding degradation.

The Rab protein family is a member of a large class of monomeric GTPases that regulate the endocytic pathway (Allen and Aderem, 1996). Investigations dealing with both pathogenic and symbiotic relationships indicate that microbes can escape digestion by interfering with phagosomal maturation. Rab5, for example, is an early endosome marker that works in conjunction with other effector proteins to promote vesicle maturation and fusion (Pfeffer, 2001). Unlike Rab5, Rab7 is not present in early endosomes; consequently, the two can be localized to different compartments of the endosome (Bottger et al., 1996). Due to its function downstream of Rab5, Rab7 plays no role in early endosome or vesicle formation (Feng et al., 1995). Feng et al. (1995) demonstrated that without Rab7 there was no late endosome formation and, as a result, an accumulation of early endosomes. Rab7 marks the appearance of late endosomes and regulates the transition to phagolysosomes (Seabra et al., 2002).

I used Western blot analysis and immunocytochemistry to test whether Rab7 could be identified in the sponge homogenate and then localized to algae-containing vacuoles of *E. muelleri*. I hypothesized that vesicles containing *Chlorella* are maintained in an immature state characterized by the absence of late endosomal markers, such as Rab7. Although Rab7 will be absent from vacuoles containing the symbiotic algae, it will be found on other vacuoles that do not contain the algae.

## Literature Review

### *The Phagocytic Pathway*

The phagocytic pathway begins with the phagocytosis of some substance from the cell's environment (Vieira et al., 2002). The entire pathway can be divided into three steps: the interaction of the particle with the receptors on the surface of the cell, the engulfing of the particle by the plasma membrane, and the formation of the phagolysosome (Desjardins et al., 1994). Phagocytosis can be defined as the uptake of large particles into cells by an actin-dependent mechanism (Aderem and Underhill, 1999). Once the particle has made contact with the surface receptors, actin is polymerized at the site of ingestion and the particle is internalized (Aderem and Underhill, 1999). The membrane that forms the phagosome is identical to that of the cell's plasma membrane, and the contents of the phagosome are like that of the extracellular medium (Vieira et al., 2002). Very soon after formation of the phagosome, the vacuole begins to undergo a series of changes brought about by fusion with other small vesicles, or endosomes, ultimately leading up to fusion with lysosomes (Vieira et al., 2002). The endocytic pathway can be viewed as "a continuum of organelles ranging from early endosomes to lysosomes" (Vieira et al., 2002).

According to Vieira et al. (2002) there are three basic groups of endosomes: the early endosomes (or sorting endosomes) the recycling endosomes, and the late endosomes. Early endosomes are designed to differentiate and route ingested materials along the endocytic pathway. Early endosomes are characterized by the presence of Rab5 and early endosome antigen 1 (EEA1). Recycling endosomes are characterized by a slightly lower pH than early endosomes and the presence of Rab11. Ingested materials designated for degradation occupy late endosomes. Late endosomes are more acidic than early and recycling endosomes and contain more hydrolytic enzymes. The appearance of Rab7 and Rab9 proteins characterizes the late endosome. The mechanism for the development of the early endosome into the late endosome is presently unknown; two models have been proposed to explain the maturation process. The vesicle shuttle model proposes that early endosomes are stable organelles. The early endosomes give rise to transport intermediaries, which then go on to become late endosomes. The maturation

model suggests that the early endosomes themselves mature into late endosomes through many fusion events. Regardless of which model is used to account for the formation of the late endosome, fusion with the lysosome to create the phagolysosome is the last step in the endocytic pathway. The lysosomes are very acidic and contain the majority of proteases and lipases.

### *Rab Protein Family*

The Rab protein family is a class of low-molecular-weight GTP-binding proteins known to participate in the membrane trafficking of the endocytic pathway (Allen and Aderem, 1996). It is believed that Rab proteins are present in all eukaryotes (Seabra et al., 2002). Rabs are known to recruit proteins (docking complexes) to the scaffolding of organelles and vacuoles (Pfeffer, 2001).

Rabs mainly associate with the cytoplasmic face of membrane bound organelles and vacuoles (Seabra et al., 2002). Rabs cycle between an active GTP-bound state and an inactive GDP-bound state (Seabra et al., 2002). When Rab proteins are in the active GTP-bound state, they recruit a group of "effector" proteins to the cytoplasmic face of the membrane (Seabra et al., 2002). Effector proteins may aid in the endocytic pathway by controlling movement, docking, and fusion of organelles (Seabra et al., 2002).

According to Novick and Zerial (1997) Rab proteins are also believed to be regulators of SNAREs, soluble NSF-attachment protein receptors. SNARE proteins are receptors for other components in the cell's cytoplasm that are necessary for docking and fusion. The SNAREs are a class of proteins distinct from other effector proteins. Rabs' relationship to effectors and SNAREs is not completely understood and clarification is needed between the roles of effectors and SNAREs. It is possible that effector proteins could serve as intermediaries between Rab proteins and SNAREs; however, it is also very likely that Rabs control both classes of proteins separately.

### *Rab7*

Rab7 has been localized to late endosomes and to lysosomes (Vieira et al., 2002). Furthermore, Rab7 has been determined not to codistribute on vesicles with the early endosomal markers Rab4 and Rab5 (Bottger et al., 1996). The separate localization of

Rab4, Rab5, and Rab7 indicates that Rab7 occupies and defines a distinct part of the endocytic pathway (Bottger et al., 1996). Additional evidence for the hypothesis that Rab7 defines a unique part of the endosomal pathway is present in the fact that internalization of the particle and endosome formation are not blocked by creating a mutant, non-viable Rab7 strain (Feng et al., 1995). The exact function of Rab7 is not currently known. Two different, but closely related, functions of Rab7 have been proposed (Vieira et al., 2002). First, Rab7 may possibly regulate the transition from early to late endosomes (Vieira et al., 2002). Secondly, Rab7 may be associated with the late endosome and regulate its fusion with the lysosome (Vieira et al., 2002). Either way, it is clear that the absence of Rab7 prevents the lysosome from delivering the degradative enzymes it contains to the endosome (Feng et al., 1995 and Seabra et al., 2002).

#### *Escaping Degradation in the Normal Endocytic Pathway*

Some pathogenic bacteria and protists enter cells and avoid degradation. Each pathogen uses a unique mechanism to avoid death within the phagosome (Allen and Aderem, 1996). Two basic categories exist for an organism's survival within a host cell: survival by exclusion and survival by stalling (Amer and Swanson, 2002). Survival by exclusion is demonstrated in many bacteria, the vacuoles of which do not acquire the transmembrane proteins that become the pumps that acidify the vacuole (Amer and Swanson, 2002). In some respects, the inability of a vacuole to acquire a membrane protein like Rab5 or Rab7 could fall under survival by exclusion; however, membrane marker protein acquisition falls under the category of survival by stalling (Amer and Swanson, 2002). Many other bacteria are known to recruit or exclude particular Rab proteins to "camouflage" their vacuole as an early endosome; in other words, they appear to stall the endocytic pathway (Amer and Swanson, 2002).

A study done with *Mycobacterium bovis* survival in macrophages indicates that the exclusion of Rab7 and the retention of Rab5 on the endosome may allow for the bacterium's prolonged existence (Via et al., 1997). The Mycobacterium-containing vacuoles were even observed to increase their levels of Rab5 (Via et al., 1997). Via et al. (1997) defined the lack of Rab7 to be the "defective checkpoint" in the maturation from early to late endosome. The lack of Rab7 was viewed as the first definite step toward the

lysosome, and its absence was viewed as the means by which degradation was halted (Via et al., 1997).

When both live and dead *Salmonella* were incorporated into macrophage endosomes, different membrane proteins were observed on their surfaces (Hashim et al., 2000). Dead *Salmonella* presented Rab7 on their late endosomes and fusion with lysosomes did occur (Hashim et al., 2000). Live *Salmonella*, on the other hand, did not acquire Rab7 and did not fuse with the lysosome (Hashim et al., 2000). Hashim et al. (2000) declared Rab7 to control late endosomes docking with the lysosome.

The parasitic protist *Leishmania donovani* provides another example of a halted degradative pathway. Like the many mycobacteria, *Leishmania* avoids degradation by blocking the acquisition of Rab7 (Scianimanico et al., 1999).

Blocking an organism's phagocytic pathways does not necessarily have a detrimental effect on the host. In cases of a symbiotic relationship being established, the host organism actually benefits from the survival of the second organism. A symbiotic relationship between a sea anemone and an alga revealed Rab7 exclusion from the phagosome to be the likely cause of the endosymbiosis (Chen et al., 2003). As was seen with the *Salmonella* in the Hashim et al. (2000) study, dead algae were degraded by the lysosomes after the late endosome displayed Rab7, while live algae were maintained in early endosomes lacking Rab7 (Chen et al., 2003). This study showed Rab7 to be crucial, not only for disease mechanisms, but also for the maintenance of a symbiotic relationship (Chen et al., 2003).

#### *Freshwater Sponges, Chlorella, and Symbiosis*

Freshwater sponges possess intricately developed canal systems of cells specialized for nutrient uptake and water transport (Hahn-Keser and Stockem, 1997). Phagocytosis is the primary process by which sponges ingest food; *Ephydatia muelleri*, specifically, displays receptor-mediated endocytosis (Hahn-Keser and Stockem, 1997). The particles are phagocytosed, processed, and then delivered to other specialized cells (Hahn-Keser and Stockem, 1997).

Many freshwater sponges are green in color due to the presence of a green symbiotic alga, like *Chlorella* (Frost and Elias, 1985). The majority of algae found in a symbiotic relationship with sponges are located within a single membrane-bounded vacuole (Williamson, 1979). All of these vacuoles originate in the digestive pathway of the sponge; however, the degradative process is interrupted to maintain the integrity of the algal cells (Williamson, 1979).

The symbiotic algae are thought to be able to influence nutrient and ionic flow across the vacuole membrane (Rands et al., 1993). It has been observed that the vacuoles housing the algae are not acidic (Rands et al. 1993). This fact argues against the hypothesis that the *Chlorella* are living in the lysosome and, instead, supports the hypothesis that the symbiotic algae are escaping the degradative pathway.

Algae-sponge symbioses are sustained by a combination of autotrophy and heterotrophy (Frost and Elias, 1985). It is not clear what factors determine the balance between the two (Frost and Elias, 1985). It appears that freshwater sponges and algae have a mutualistic symbiosis (Frost and Elias, 1985). Mutualism benefits both species as it increases the efficiency of gathering nutrients (Frost and Elias, 1985). The alga is not dependent on its host, but it does receive protection from the sponge (Saller, 1991). The sponge, in return, receives the product of photosynthesis, glucose (Saller, 1991). Although it is difficult to measure exactly how much nourishment the sponge receives from the algae, it is thought that the sponge benefit from the relationship enough to be able to grow substantially larger (Sand-Jensen and Pedersen, 1994).

## Materials and Methods

### *Sponge Collection*

*E. muelleri* was collected from two locations in Montana, South Fork Lake (46°17' N/111°50' W), Jefferson County and Coopers Lake (47°05' N/112°55' W), Powell County. The sponges were transported in lake water to the lab where they were immediately dissociated.

### *Dissociation of Cells*

About 3 mL of CMF (calcium/magnesium free medium: 6mM Tris-HCl + 34mM NaCl + 1.34 mM KCl + 1.38 mM glucose + 0.7 mM K<sub>2</sub>HPO<sub>4</sub> + 0.25 mM EDTA, pH 7.6) was added to 2-3 grams of sponge. As much debris was removed from the sponge as possible. The sponge was minced with a razor blade and transferred to a test tube. The total volume was brought to 5 mL and then left to sit at room temperature for 5 min. The slurry was then vortexed on the 7.5 setting of a FisherBrand Genie 2 vortex for 5s at least seven times. To remove the spicules and any remaining debris, the slurry was filtered through 120µm Nitex mesh. Finally, the slurry was transferred to centrifuge tubes and centrifuged at 1000g for 15s. The resulting supernatant was discarded and the pellet was used in the following homogenate and cell suspension.

### *Homogenate and Cell Suspension Preparation*

For use in the Western blot analysis, the pellet samples were re-suspended with 50µL PBS-LS (50 mM NaCl + 5 mM NaPO<sub>4</sub>, pH 7.2). The pellet/PBS-LS suspensions were then transferred to a homogenization tube. The suspension was homogenized at setting 8 of a Bellco homogenizer with 8 up-down strokes on the pestle. The homogenate was then transferred to a microtube for later use. The homogenate was refrigerated for immediate use or frozen if it was not used immediately.

For immunocytochemical analysis, the pellet was re-suspended with as little PBS-LS as was needed for re-suspension. The mixture was transferred to a microtube and enough Polyfreeze was added to compose about two-thirds of the total volume. The suspension was stirred to evenly distribute the dissociated sponge cells throughout the Polyfreeze and then the cell suspension was frozen.

### *Western Blot Analysis*

The protein concentration of the homogenate was determined using the Bradford assay and a BSA standard (bovine serum albumin). The proteins were separated by SDS PAGE in 12% gels. Enough of the homogenate was added to have between 30 and 50 $\mu$ g of protein loaded in the lane. Four  $\mu$ g of Sigma Dalton Marker VII and 15 $\mu$ g of mouse brain homogenate were electrophoresed. The mouse brain homogenate served as a positive control for protein detection.

After electrophoresis, the gels were placed in a glycerol wash (20 mL glycerol + 80 mL of 0.6g Tris HCl, pH 7.5) and rocked for two 10 min intervals.

Using a renaturing transfer buffer (10mM NaHCO<sub>3</sub> + 3mM Na<sub>2</sub>CO<sub>3</sub>), the proteins were recovered by transfer to nitrocellulose membranes. After the transfer, one of the membranes was stained for protein using amido black stain (0.1% (w/v) amido black in 45% methanol + 10% ethanol). The membrane was placed in the solution until bands were visible. The membrane was then placed in a destaining solution of 90% methanol and 2% acetic acid. Finally, the membrane was placed in water and placed on filter paper to dry.

The second membrane was placed in a Press-And-Seal bag with PBS/Blotto (5% [w/v] nonfat dry milk in 0.15 M NaCl + 10 mM NaPO<sub>4</sub>, pH 7.4 + 0.05% Tween 20) overnight in the refrigerator. Next, it was incubated in a 1:25 or 1:10 anti-human Rab7 antibody (Santa Cruz) in PBS/Blotto solution for 1 h. It was transferred to a clean Press-And-Seal bag and washed with PBS/Tween 20 (0.15 M NaCl + 10 mM NaPO<sub>4</sub> + 0.05% Tween 20) 4 times, 3 min each. It was then incubated in a solution of 1:3000 alkaline phosphatase anti-rabbit IgG antibody conjugate (Sigma) in PBS/Blotto solution for 1 h. The membrane was transferred to another clean bag and washed with PBS/Tween 20 for 5 min. It was washed twice with water for 5 min each and incubated in BCIP/NBT (Sigma) solution until the banding was visible.

### *Immunocytochemistry*

The frozen cell suspension for immunocytochemistry was sectioned using a cryostat. The sections were affixed to gelatin coated slides by melting them onto the

slides. The slides were then placed in a humidifying chamber and covered with PBS for 5 min. Next the slides were covered with a 0.2% Triton X-100/PBS solution for 10 min to make the cell membranes permeable. The slides were then fixed with 3% formaldehyde in PBS for 15 min. The formaldehyde was poured off and the slides were covered with 0.5% saponin in PBS twice for 5 min each. Next, the slides were covered with 0.5% saponin and 10% goat serum in PBS 3 times for 5 min each to block non-specific protein binding. Each experimental slide was then incubated in a 1:10 anti-Rab7 in 0.5% saponin/10% goat serum/PBS solution for 1 h. The primary antibody was rinsed off the slide with this solution 3 times for 5 minutes each. Both the experimental and control slides were incubated in a 1:100 alkaline phosphatase anti-rabbit conjugate (Sigma) in 0.5% saponin/10% goat for 30 min. The slides were then rinsed 3 times in PBS for 10 min each. The slides were incubated in the Fast Red/Naphthol As-MX (Sigma) color solution for 2-3 min. Finally, the slides were rinsed 3 times in PBS for 2 min each rinse. The slides were covered with a mounting gel and coverslip and kept in a refrigerator until observed. The slides were observed by bright field microscopy and photographed.

Alternatively, the slides were fixed with the 3% formaldehyde in PBS solution before they were covered with the 0.2% Triton X-100 solution. This was done in the hope of securing the cells to the slide before making their membranes permeable.

## Results

### *Western Blot*

Western blots were performed on the sponge homogenate to determine whether the antibody directed against human Rab7 could cross link with the sponge Rab7. The results were inconsistent. Ten Western blot analyses were conducted using the Rab7 antibody. Of the ten experiments, I will discuss the last two, as they are representative of the entire data set. With a 1:25 antibody dilution only extremely faint bands were visible in the lane containing the sponge homogenate (Fig. 1). Neither of the bands was near the expected molecular weight for Rab7. With a 1:10 antibody dilution, I observed four very faint bands (Fig. 1). Here, however, the band with the lowest molecular weight was in the expected range for Rab7.

### *Immunocytochemistry*

Immunocytochemistry was performed to localize Rab7 in sponge cells containing symbiotic algae. Two experiments were performed, producing inconsistent results. Under one set of conditions (permeabilization preceding fixation), the sponge cells incubated in the Rab7 antibody had a diffuse red color to their cytoplasm (Fig. 2). Some other small points, possibly other vesicles devoid of algae, also stained red. These localized points of staining were not conclusively identified. There was no appearance of reaction product (red color) on the vacuoles containing the algae. The control showed no detectable staining (Fig. 3).

Under a second set of conditions (permeabilization after fixation), however, the results differed. Rather than observing a diffuse red color in the cytoplasm of the vacuole containing cells stained for Rab7, I observed no red staining (Fig. 4). Again, the control slide was free of staining (Fig. 5).

In contrast to the results obtained with anti-Rab7 antibodies, antibodies against human Rab5 displayed a prominent staining pattern (results not shown). The cytoplasm of the cells incubated in anti-Rab5 was less red than those stained with Rab7. However, the algal-containing vacuoles showed prominent staining on their exterior, indicating an antibody-protein cross reaction and the presence of Rab5. The Rab5 experiment demonstrated a positive result for vacuole staining, which the Rab7 clearly did not possess.

## **Discussion**

### *Western Blot*

When the antibody was used in a 1:25 dilution, no staining was seen in the proper range of molecular weights for Rab7. The absence of Rab7 could be due to several factors. First, the concentration of Rab7 in the sample of sponge protein may have been too small to be detected. Secondly, the anti-human Rab7 antibody I used may not have been able to sufficiently cross react with the sponge Rab7 protein. Lastly, it is possible that the denaturation of the Rab7 protein, which occurred during SDS-PAGE, was irreversible. However, this possibility seems unlikely in view of the positive Western blot

results obtained by others using the same or similar procedures and studying very similar systems (Chen et al., 2003; Jarvis, 2005).

With a 1:10 antibody dilution, however, an extremely faint band was seen in the proper range of molecular weights for Rab7. The band's faintness may also be attributed to the scarcity of the protein in the sample and/or the antibody's lack of cross-reaction with the sponge protein. If the faint band is interpreted to be a positive result, the success could be attributed to the increased antibody concentration. It is possible that the more concentrated antibody was able to stay bound to the small amount of protein present. Increasing the antibody concentration produced a further complication that makes this explanation unlikely. Two of the bands visible in the 1:10 antibody dilution were not present in the 1:25 dilution. The banding inconsistency only increases the questions concerning antibody specificity for sponge protein.

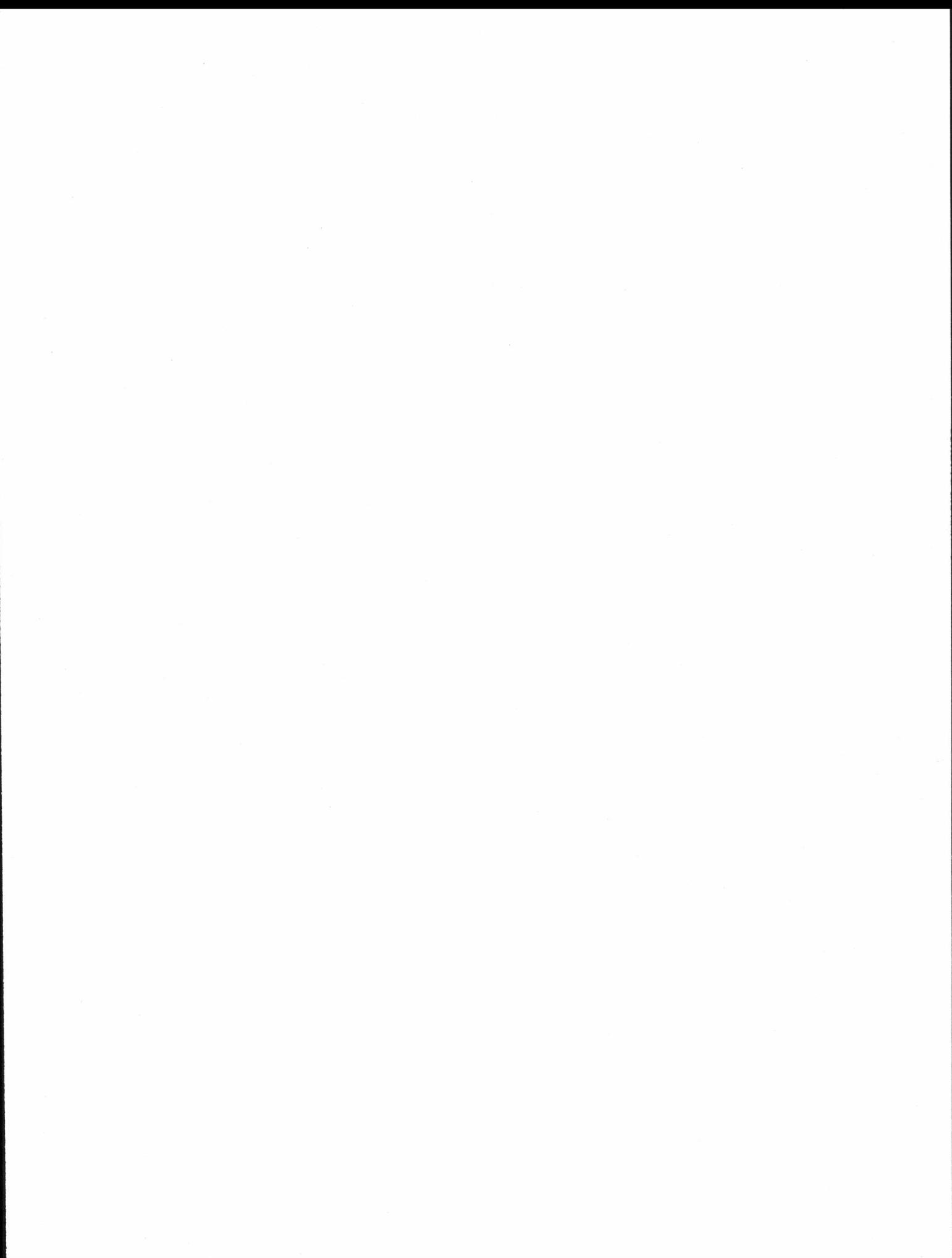
#### *Immunocytochemistry*

The two experiments produced conflicting results, one positive for staining and the other without staining. The difference in results may be attributed to one of two factors. First, the change in conditions, although small, may have caused the discrepancy. In the second immunocytochemistry procedure two steps were reversed, the cells were fixed with the 3% formaldehyde in PBS before they were covered in the 0.2% Triton X-100 solution. Secondly, the Rab7-antibody cross reactivity may be weak, a factor made more problematic by the possibility that Rab7 may not be present in large amounts in the sponge cell.

If, in fact, the staining pattern observed in the first experiment, accurately reflects the distribution of Rab7 in the sponge cells, then it appears likely that phagosomal maturation is blocked before Rab7 acquisition. From the lack of Rab7 acquisition, it can be inferred the vacuoles never reach the late endosomal stage. Additionally, this result suggests that surface marker proteins are the vehicles for halting the degradation pathway.

### **Conclusions**

The lack of consistency in the Western blots and immunocytochemical results are troubling and do not allow me to make definite conclusions. It is possible that a Rab7 antibody directed against nonhuman or non-mammalian Rab7 would be more effective in reacting with the sponge protein. Future researchers may also consider studying a completely different late endosome marker protein and using the corresponding antibody.



## Figures

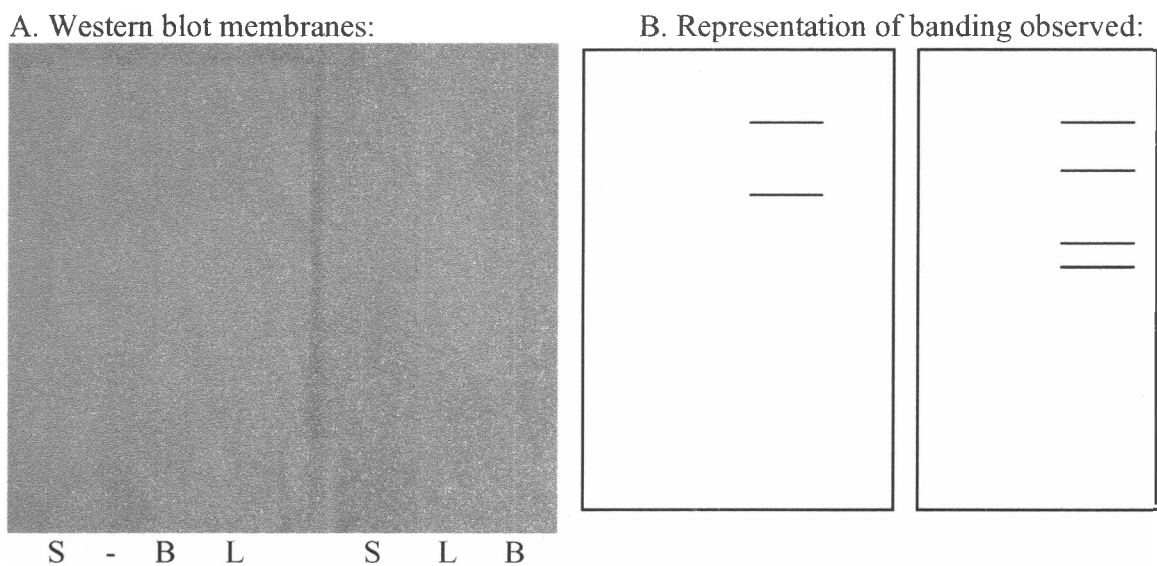


Figure 1: A. Western blot results. Membrane on the left was incubated in a 1:25 anti-Rab7 solution. Membrane on the right was incubated in a 1:10 anti-Rab7 solution. Banding is extremely faint and not visible in photograph. B. The drawings indicate where banding was observed in both the 1:25 and 1:10 dilutions respectively.

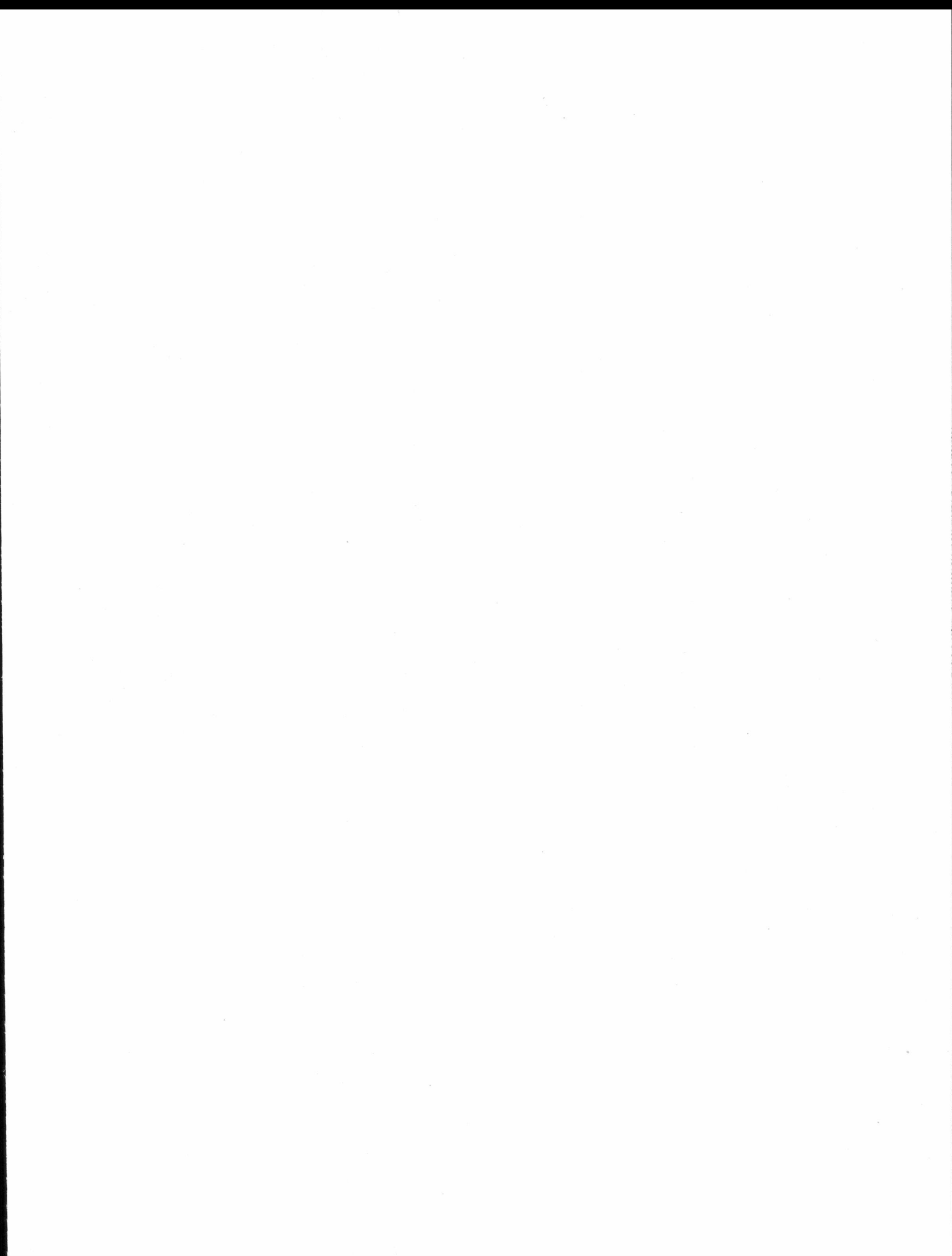




Figure 2: Result of first Rab7 localization (permeabilization precedes fixation). Diffuse staining is visible in the sponge cell. Vacuoles containing the algae (green) are unstained.

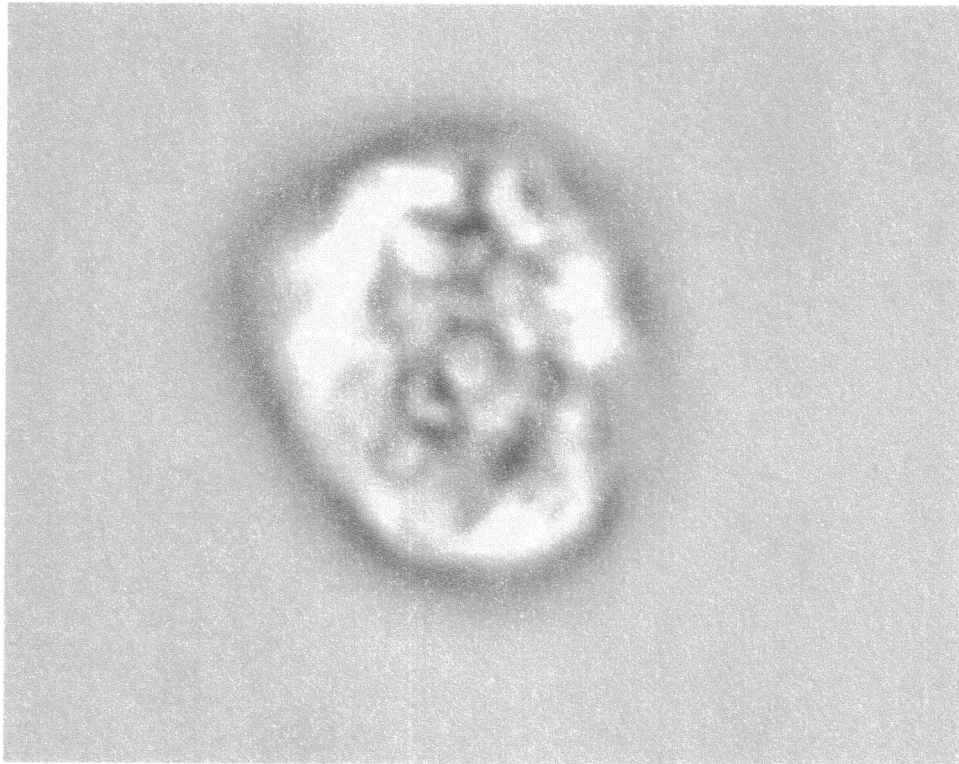
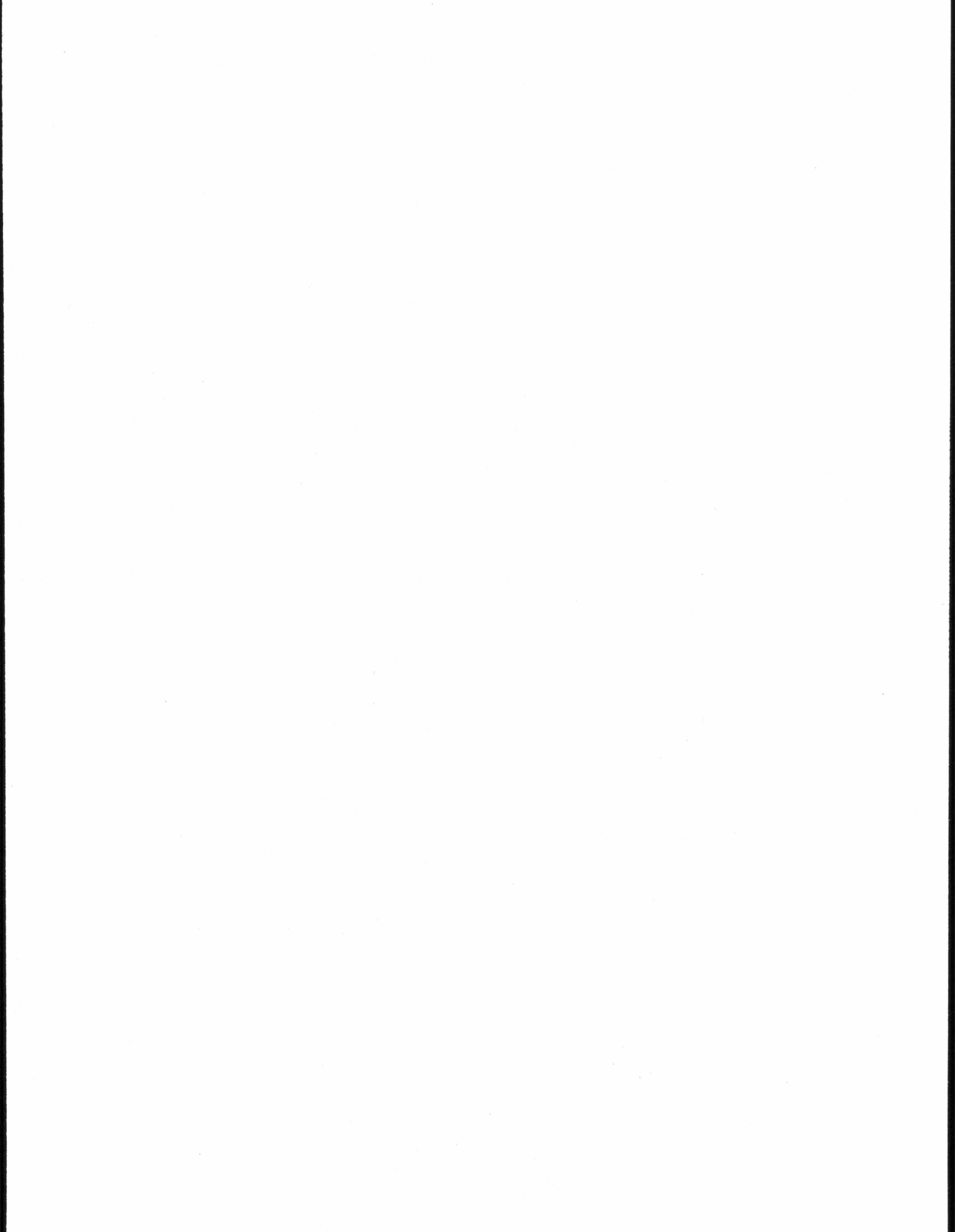


Figure 3: Control sponge cell from first localization procedure (permeabilization precedes fixation). Control cell is without any visible staining.



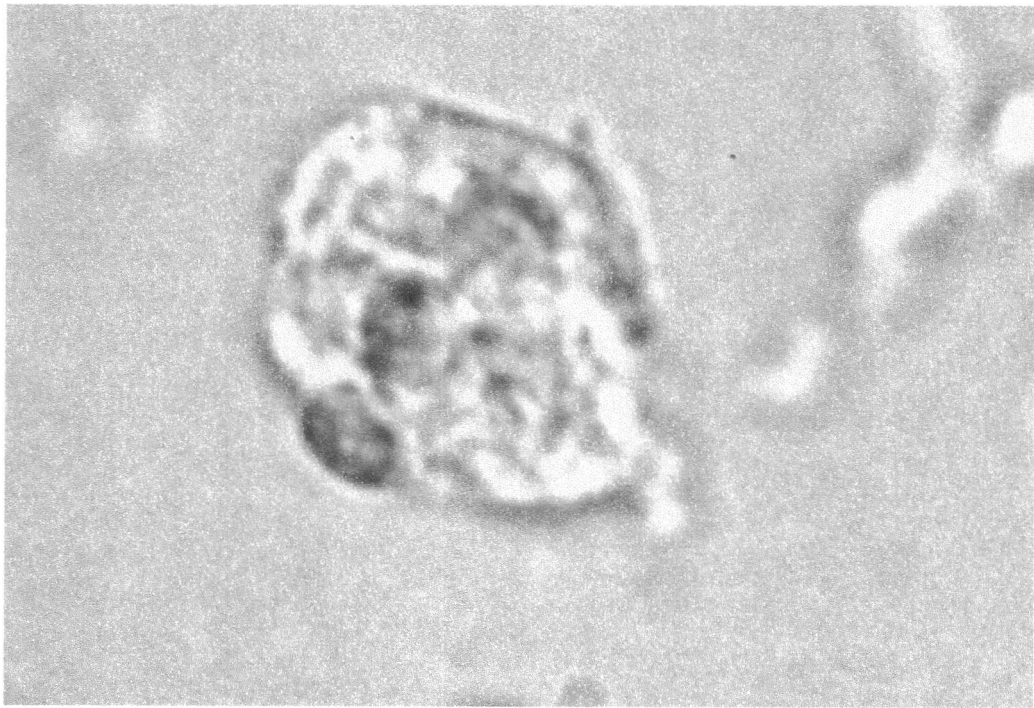
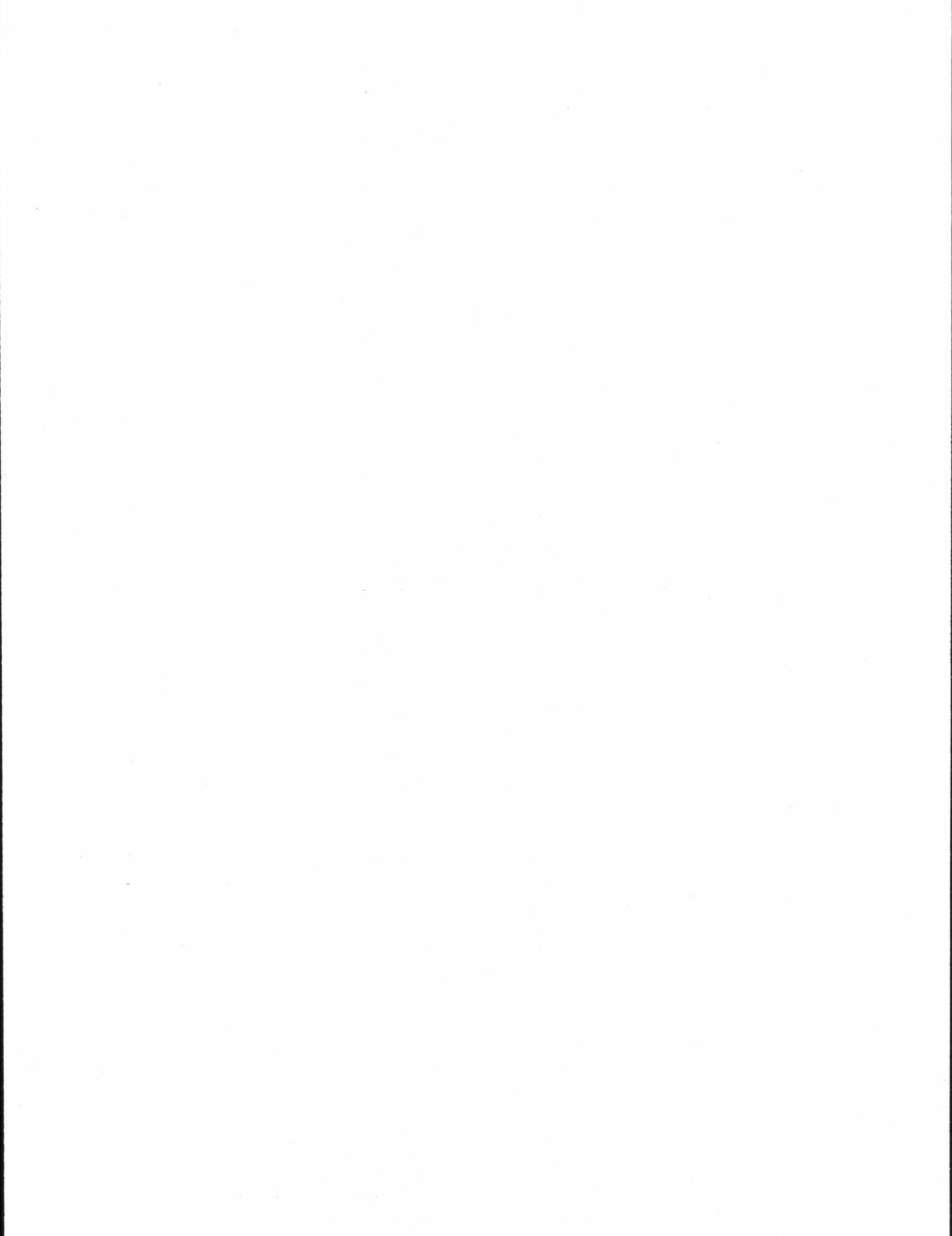


Figure 4: Localization of Rab7 in sponge tissue using the second procedure (fixation precedes permeabilization). Experimental sponge cell is without any observable staining.



Figure 5: Control sponge cell from second localization procedure (fixation precedes permeabilization). Control cell is without any visible staining.



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