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Phagosomal Proteins of *Dictyostelium discoideum*

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Abstract. In recognizing food particles, *Dictyostelium* cell-surface molecules initiate cytoskeletal rearrangements that result in phagosome formation. After feeding *D. discoideum* cells latex beads, early phagosomes were isolated on sucrose step gradients. Protein analyses of these vesicles showed that they contained glycoproteins and surface-labeled species corresponding to integral plasma membrane proteins. Cytoskeletal proteins also were associated with phagosomes, including myosin II, actin and a 30 kDa-actin bundling protein. As seen by the acridine orange fluorescence of vesicles containing bacteria, phagosomes were acidified rapidly by a vacuolar H^+-ATPase that was detected by immunoblotting. Except for the loss of cytoskeletal proteins, few other changes over time were noted in the protein profiles of phagosomes, suggesting that phagosome maturation was incomplete. The indigestibility of the beads possibly inhibited further endocytic processing, which has been observed by others. Since nascent phagosomes contained molecules of both the cytoskeleton and plasma membrane, they will be useful in studies aimed at identifying specific protein associations occurring between membrane proteins and the cytoskeleton during phagocytosis.

Keywords: Actin, cytoskeleton, phagocytosis, vacuolar H^+-ATPase

Phagocytosis, the engulfment of large particles greater than 1 mm in diameter, begins with the recognition and binding of a particle by receptors found on the surface of phagocytes such as polymorphonuclear leukocytes, macrophages and free-living amebae [22]. Particle binding triggers cytoskeletal rearrangements including actin recruitment and polymerization at the plasma membrane that result in pseudopod formation [22]. After particle internalization, the newly formed vesicle, called a phagosome, is rapidly acidified by a proton-pump ATPase [28, 30] and merges into the lysosomal/endosomal compartment where hydrolytic enzymes bring about the digestion of the particle. Cell-surface receptors eventually are recycled [2, 12, 36, 41]. Although a number of phagocytosis-promoting receptors, such as Fc receptors [44] and complement receptors [57], have been identified, aspects of the signaling pathway linking the receptor to actin polymerization are still emerging [1, 20, 21].

We are asking how the cell-surface is linked to the actin-based cytoskeleton during phagocytosis using the cellular slime mold *Dictyostelium discoideum. D. discoideum* amebae are active phagocytes capable of ingesting bacteria, yeast and mock food particles such as latex beads [7, 9, 16, 54, 58] in a manner that parallels the phagocytosis process in polymorphonuclear leukocytes and macrophages. The ability to perform biochemical and molecular genetic analyses of *D. discoideum* makes this organism an effective system for the examination of membrane-cytoskeletal interactions that occur in the early stages of phagocytosis.

To begin identifying molecules with roles in the initial steps of phagocytosis, our approach was to establish the protein profile of phagosomes isolated from *D. discoideum* cells fed latex beads. These vesicles were expected to contain both plasma membrane proteins that potentially could function as receptors and cytoskeletal proteins that had an immediate and relatively stable association with the membrane during phagosome formation. When compared to separately isolated plasma membranes and cytoskeletons, we found that phagosomes isolated 10 min after ingestion contained a subset of proteins that reflected the interface between the cell-surface and the actin-based cytoskeleton. Specific plasma membrane glycoproteins were not enriched noticeably in phagosomes, indicating that internalized membranes contained largely a random representation of the cell-surface. Cytoskeletal proteins such as actin, myosin and an actin-bundling protein were identified in early phagosomes.

Protein profiles of phagosomes isolated thirty and sixty minutes after ingestion showed reduced levels of cytoskeletal proteins, increased levels of a proton pump-ATPase and slightly elevated levels of Concanavalin A (Con A)-binding proteins over time. The acidification of phagosomes was observed and likely due to the addition of a vacuolar H^+-ATPase and fusion with prelysosomal vesicles or lysosomes. Decreased levels of Con A-binding proteins that corresponded to plasma membrane proteins were not observed, suggesting that the use of latex beads may have altered the normal recycling of membrane proteins to the cell-surface.

Materials and Methods

Phagosome isolation. *D. discoideum* cells (AX3-K) were grown to 6–8 × 10^6 cells/ml at 20°C in HL5 media [55]. Cells were centrifuged at 500 × *g* for 2 min, washed twice in cold Sorensen's buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.1), and resuspended to 5 × 10^6 cells/ml in cold buffer. Cells were chilled deliberately to reduce the rate of phagocytosis. Polystyrene beads (washed twice in Sorensen's buffer; 100 beads/cell; 1.007 μm diameter; Polysciences, Warrington, PA) serving as a pulse of food particles, were added to the cell suspension, which was then shaken (125 rpm) at 20°C. After 10 min, the cell suspension had warmed to 10°C, and the phagosomes isolated after this short interval of shaking potentially would contain molecules involved early in their formation. Cells were diluted with two volumes of cold Sorensen's buffer to stop phagocytosis. All subsequent manipulations were done at 2–4°C. Cells were harvested by centrifugation at 500 × *g* for 2 min and washed once with two volumes of cold Sorensen's buffer before being resuspended to 5 × 10^7 cells/ml in cold homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 10 mM benzamidine hydrochloride, 2 mM phenyl methyl sulfonyl fluoride, 1 mM EDTA, 2 mM leupeptin, pH 7.6). The dilution and washing of cells removed most of the uningested and beads adhering to cell surfaces. By microscopy, we estimated that greater than 90% of the beads were inside cells, and consequently, surface-bound beads did not contribute significantly to the protein profiles observed. Phagosomes were released by filter lysis of cells (5 mm pore size, COSTAR Nucleopore, Cambridge, MA) [43]. Fresh phenyl methyl sulfonyl fluoride was added to a final concentration of 2 mM to the cell lysate before centrifugation, Cell lysates were centrifuged at 500 × *g* for 2 min to pellet unbroken cells and nuclei. To the post-nuclear supernatant (PNS), an equal volume of 60% (w/v) sucrose in 3 mM imidazole, pH 7.4, was added [12].

Samples were layered onto a 2-ml cushion of 62% sucrose, and overlaid with 6 ml of 35% sucrose, 6 ml of 25% sucrose, and 6 ml of 10% sucrose. All sucrose solutions were prepared in 3 mM imidazole, pH 7.4 and chilled. Gradients were centrifuged at 100,000 × *g* for 1 h at 4°C (SW28 swinging bucket rotor; Beckman Instruments, Palo Alto, CA). The phagosome fraction was collected from the 10–25% interface and washed twice (39,000 × *g* for 20 min) with 20 mM sodium phosphate, pH 6.8 ($NaPO_4$ buffer). The pelleted phagosomes were resuspended in a small volume of $NaPO_4$ buffer, 1% SDS, and heated at 80°C for 10 min.

Equivalent numbers of cells also were lysed in the absence of beads and the PNS loaded onto parallel sucrose step gradients. In three separate experiments, the 10–25% interface contained only 0–7% of the protein found at the 10–25% interface of experimental gradients. This indicated that protein found at this interface was due to interactions with the latex beads. In an additional control experiment, cells were fed beads and then washed. Cells that were metabolically radio-labeled separately were added to the bead-fed cells, and the cells were jointly lysed. There was little radioactivity associated with the isolated phagosomes, indicating that protein from the cell lysate did not become associated nonspecifically with the latex beads once phagosome formation had occurred.

To prepare 30- and 60-min phagosomes, cells ingested beads for 10 min in cold Sorensen's buffer, and then were pelleted at 500 × *g* for 2 min. Cells were resuspended to 5×10^7 cell/ml in cold Sorensen's buffer, chilled on ice for 10 min, and then diluted with nine volumes of Sorensen's buffer at room temperature. After shaking for either 30 or 60 min, cells were harvested and resuspended in homogenization buffer. Cell lysis and collection of phagosomes were as described above. All phagosome preparations were repeated a minimum of three times. A second protocol was used to isolate phagosomes. *D. discoideum* cells (2.5×10^7 cells/ml) were allowed to take up latex beads (0.8 μm diameter, blue-dyed; Sigma Chemical Co.; St. Louis, MO) at 200 beads/cell in HL5. Cells were shaken (150 rpm) at 18°C, and washed in HL5 by centrifugation (1,000 × *g* for 3 min) three times. Internalization and chase times indicated in the legend for Figure 5 were performed at the same cell density and conditions. Cells then were washed once with homogenization buffer (100 mM sucrose, 5 mM glycine, pH 8.5) and resuspended at 2×10^8 cells/ml in the same buffer before lysis by passage through double filters (5 μm pore size). The lysate was centrifuged at 1,000 × g for 3 rnin to remove unbroken cells and nuclei. The PNS was brought to 40% sucrose by addition of 62% sucrose solution (sucrose solutions were wt/wt in 5 mM glycine, pH 8.5) and loaded onto a 1-ml cushion of 62% sucrose. Seven ml each of 35%, 25% and 10% sucrose solutions were then successively added, and gradients were centrifuged as described above. These buffer conditions favored the partial retention of the 70-kDa subunit that is part of the peripheral domain of a vacuolar H^+-ATPase, whereas buffers at a lower pH caused almost a complete loss of the 70 kDa subunit from phagosomes. The association of the 41-kDa subunit of the vacuolar H^+-ATPase with phagosomes appeared largely unaffected by the different buffers used to lyse and subsequently isolate phagosomes (unpublished observations). Phagosomes collected from the 10–25% sucrose interface were washed in 100 mM sucrose, 5 mM glycine, pH 8.5 (40,000 × *g* for 40 min) before the addition of SDS and heated at 65°C for 10 min.

To rule out the possibility that the 30-kDa-actin-bundling protein (30 kDa-ABP) was binding nonspecifically to phagosomes, 100 mg of a myc-tagged form of the 30 kDa-ABP (provided by Dr. M. Fechheimer, University of Georgia, Athens) was added immediately after cell lysis in a control experiment. Ten-minute phagosomes were prepared as described. Also, to an aliquot of the PNS, SDS was added to 1%, and heated at 80°C for 10 min. Immunoblots of phagosomes and the PNS were probed with a monoclonal antibody that recognizes the human c-myc protein (American Type Culture Collection, Rockville, MD; [13]). Bound anti-myc antibody was detected with a goat antimouse IgG, IgM antibody conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, IL). Signals from phagosomes and PNS were compared to signals of known amounts of myctagged protein, and the amount of myc-30 kDa-ABP associated with phagosomes was estimated.

Phagosomes from surface-labeled cells. Cells were harvested and washed once in cold Sorensen's buffer as described above. Cells then were washed in cold Sorensen's buffer, pH 8.3, resuspended to 1×10^8 cells/ml and sulfo-NHS-biotin (Pierce) was added to 200 mg/ml [19]. After shaking (150 rpm) for 30 min on ice, cells were pelleted, washed with excess cold Sorensen's, pH 6.1, and resuspended to 5×10^6 cells/ml. Ten-minute phagosomes then were prepared as described.

Plasma membrane and cytoskeleton preparations. Plasma membranes (PM) were prepared as described by Das and Henderson [43]. Cytoskeletons were prepared as described by McRobbie and Newel1 [31] in the presence of 5 mM benzamidine, 2 mM phenyl methyl sulfonyl fluoride, and 1 mg/ml leupeptin.

NaOH-treated phagosomes and PM. Ten-minute phagosomes and PM samples were treated with NaOH to remove peripheral membrane proteins [46]. Species that remained associated with pelleted membranes were designated as integral proteins. Approximately 5 mg of phagosomes or PM were added to cold 100 mM NaOH, containing 1.1 mM dithiothreitol in a chilled polypropylene tube on ice. The chilled mixture was sonicated with a probe sonicator (Vibra Cell, Sonics and Materials Inc., Danbury, CT) for 10 sec every 10 min at 25% maximum output for 30 min. Treated-membranes were washed twice with $NaPO_4$ buffer (38,720 × *g* for 20 min) and resuspended in a minimal volume of $NaPO_4$ buffer

Protein analyses. Protein was assayed with the Lowry method [27] in the presence of 0.05% SDS using bovine serum albumin (BSA) as a standard. Enzyme assays were performed following previously published procedures [4]. Protein composition was analyzed using the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli [25]. Gels were either Coomassie Blue-stained [14], silver-stained [33], or electrophoretically transferred to nitrocellulose (0.5 μm pore size; Schleicher and Schuell, Keene, NH) [53] using a tank apparatus. Prestained molecular mass standards (lysozyme, 15 kDa; P-lactoglobulin 18 kDa, carbonic anhydrase, 28 kDa; ovalbumin, 44 kDa; BSA, 71 kDa; phosphorylase B, 105 kDa; myosin [Heavy chain], 216 kDa) were obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Relative masses were calculated from a standard curve created by plotting the log of the molecular mass of standards versus their mobility.

Glycoproteins were detected by probing blots with 10 μg/ml biotin-concanavalin A (Con A; Sigma) or 100,000 cpm/ml ^{125}I-Con A (Na-^{125}I, ICN Pharmaceuticals, Costa Mesa, CA) [5] in 20 mM Tris, 150 mM NaCl, 0.02% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2% bovine serum albumin [19]. Blots also were probed with 10 μg/ml biotin-wheat germ agglutinin (WGA; Sigma) or ^{125}I -WGA (100,000 cpm/ml) in the same buffer [19]. Con A- and WGA-binding proteins were visualized with streptavidin-horseradish peroxidase (Pierce) and chemiluminescence (Amersham ECL, Arlington Heights, IL), or by autoradiography (Kodak X-OMAT film; Eastman Kodak Co., Rochester, NY). Blots of surface (biotin)-labeled proteins were probed with streptavidin-horseradish peroxidase (5 μg/ml; Pierce) and signals were visualized by chemiluminescence. To verify the co-migration of glycoproteins with surface-labeled proteins, the same blots were stained with different probes. For example, blotted surface (biotin)-labeled proteins were identified with streptavidin-horseradish peroxidase and developed with chemiluminescent substrates. Blots then were washed and probed

Table 1. Relative yield of enzyme activities[a] and protein of isolated phagosomes.

	Acid phosphatase	Alkaline phosphatase	α-Glucosidase II	Protein
% of total homogenate[b]	2.9	0.5	0.35	0.79
Relative specific activity	3.7	0.63	0.44	

[a] See Materials and Methods for enzyme assays.
[b] Mean of three experiments.

with ^{125}I-Con A. Signals of double-probed blots were identical to single-probed blots. The specificity of lectin binding was determined by incubating duplicate blots with 200 mM a-methy1 mannoside for Con A blots or 200 mM N-acetylglucosamine for WGA blots before the addition of lectin.

Protein blots were probed with antibodies that recognized the 30 kDa-ABP (provided by M. Fechheimer, University of Georgia), actin (Sigma), myosin II heavy chain (Dr. S. Ravid, Hebrew University, Jerusalem), affinity-purified antibody to gp130 [6] or the 41-kDa subunit of the *D. discoideum* vacuolar H^+-ATPase [49]. Antibodies to the 70-and 100-kDa subunits of the *D. discoideum* vacuolar H^+-ATPase were provided by Dr. A. Fok (University of Hawaii) [15]. Bound antibody was detected colorimetrically using the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Amresco, Solon, OH) [26] and a goat antirabbit IgG alkaline phosphatase conjugate (Pierce). Control blots were probed solely with goat anti-rabbit IgG alkaline phosphatase to determine nonspecific binding of the secondary antibody.

Signals on gels, blots and X-ray films were quantified by densitometry using the UVP GelBase Program (Ultra Violet Products, Cambridge, UK). Signals are expressed as a percentage of the combined total area of the bands examined and are the average of three different preparations.

Microscopy. Bacteria labeled with fluorescein isothiocyanate (FITC) [54] were added to cells growing in HL5 at a final ratio of 50 bacteria per cell. After a 15-min incubation, cells were collected and washed free of bacteria by repeated differential centrifugation. Acridine orange was added to a final concentration of 3 μM [38] and cells were examined using an Olympus fluorescence microscope equipped with barrier filters to separately visualize FITC and acridine orange fluorescence.

Results

Early phagosomes possessed proteins similar to that of the PM. Phagosomes were isolated from *D. discoideum* cells by adapting a previously described method that exploited the buoyancy of ingested latex beads on sucrose gradients [12, 56]. To determine the purity of isolated *D. discoideum* phagosomes, assays were done to detect the presence of lysosomal acid phosphatase, contractile vacuole alkaline phosphatase and endoplasmic reticulum (ER) a-glucosidase, all of which are soluble marker enzymes for the different membrane-bound compartments that potentially could co-isolate with phagosomes. As indicated in Table 1, 2.9% of the total acid phosphatase activity and 0.79% of the total protein in the homogenate was recovered in purified phagosomes prepared from cells fed beads for 15 min. The relative increase in acid phosphatase specific activity in 15-min phagosomes therefore was almost fourfold. In contrast, less than 1% of alkaline phosphatase and α-glucosidase was recovered in purified phagosomes and the final relative specific activity for these enzymes was less than one (Table 1). These results indicate that lysosomal acid phosphatase was enriched in 15-min phagosomes while contamination of phagosomes with elements of the contractile vacuole or ER microsomes was minimal.

Since phagosome membranes are derived from the PM, similarities in their protein profiles were expected. Proteins from 10-min (early) phagosomes (EP) and PM were compared by SDS-PAGE (Figure 1). Coomassie Blue-stained gels showed the co-migration of EP and PM species at 194, 170, 152, 130, 105 kDa (Figure 1A). Other species, including ones at 75, 50 and 48 kDa also appeared to co-migrate, although their relatively weak staining in PM precluded their unequivocal alignment. Although lanes were loaded with equal amounts of protein, Coomassie Blue-stained gels containing PM typically appeared to be underloaded compared to EP. This may be due to the reduced amount of Coomassie Blue bound to the glycoproteins in PM fractions [18]. Positive identification of prominent Coomassie Blue-staining proteins in EP such as myosin II heavy chain (at 215 kDa, m) and actin (at 43 kDa, 43a) (see Figure 3), was performed by immunoblotting. The 43 kDa protein in PM did not react with actin antibodies (data not shown). A 30-kDa actin-bundling protein (30 kDa-ABP) shown to associate with phagocytic cups [16], also was evident in EP (see Figure 3). Glycoproteins (gp) of EP were identified by their ability to bind Con A or WGA, as revealed by lectin staining of protein blots (Figure 1B, C). EP contained Con A-binding proteins that co-migrated with Con A-binding proteins of the PM at 194, 152, 130, 105, 75, 70 and 65 kDa (lanes 3 and 4). Signals at 48, 43, 36, and 32 kDa, detected in EP, were seen in PM at longer exposures (data not shown). Only two WGA-binding species at 180 (gp180) and 166 kDa (gp166) were detected in both PM and EP (Fig 1C). Unlike their counterparts in PM (lane 5) which had relatively similar signal intensities, gp166 in EP consistently bound less WGA than gp180 (lane 6). Possibly, gp166 was not internalized during phagocytosis or removed rapidly from the phagosome membrane.

To identify surface-exposed species shared by PM and EP, intact cells were labeled before isolating cell fractions with a membrane-impermeant biotinylating reagent reactive with primary amino groups. The profile of biotin-labeled proteins in EP was consistently similar to that of PM (Figure 1D). Thirteen biotin- labeled proteins common to EP and PM were observed at 200, 194, 160, 130, 110, 105, 85, 70, 68, 65, 44, 43 and 41 kDa. An additional five faint signals, at 38, 35, 32, 20, and 18 kDa in EP, co-migrated with PM signals. A species at 73 kDa observed in EP but not in PM may have reflected a slight enrichment of this molecule in EP (marked with a *; lane 7) compared to PM (lane 8). This was not a cytosolic protein because labeling of actin and myosin, proteins that also would have been biotinylated if some cells had lysed in the presence of reagent, was not observed (data not shown). In contrast, a strongly labeled species at 17 kDa in PM appeared to be absent from EP. Seven surface-labeled proteins, at 194, 130, 105, 70, 65, 43 and 32 kDa, had molecular masses corresponding to Con A-binding proteins shared by EP and PM. The other surface-labeled proteins did not co-migrate with observed Con A- or WGA-binding proteins. These biotinylated proteins either were not glycosylated or, in the case of the lower molecular weight species (< 40 kDa), it appeared that the chemiluminescent detection method was more sensitive than autoradiography with iodinated lectins. Like the glycoprotein patterns, the similarity of the biotin-labeled protein profiles between PM and EP indicated that many of the EP proteins were derived from the PM.

EP and PM had corresponding integral membrane glycoproteins. We were interested in identifying integral membrane proteins (IMP) because of their potential role in linking the cell-surface to the cytoskeleton. Peripheral proteins were removed from PM and EP preparations using an NaOH treatment. We found that IMP of EP co-migrated with IMP of PM (Figure 2). Silver-stained SDS-gels showed

that at least 9 of 15 IMP of EP co-migrated with IMP of PM (data not shown). These nine co-migrating IMP-bound Con A (Figure 2, lanes 2 and 4), three of which, gp170, gp85 and gp68, were previously undetected on Con A blots (Figure 1B), although an 85 kDa species were observed by surface-labeling (Figure 1D). Their detection likely was due to their increased relative concentration in the treated samples depleted of peripheral proteins.

EP contained cytoskeletal proteins and cytoskeletally-associated glycoproteins. Since the cytoskeleton has been shown to play a critical role in the engulfment step of phagocytosis [22], EP were expected to contain cytoskeletal proteins responsible for pseudopod extension. Comparison of EP and detergent-insoluble cytoskeletons on Coomassie Blue-stained gels showed that at least ten species of EP co-migrated with proteins found in cytoskeletons (indicated in Figure 3A). Myosin II heavy chain (215 ma), actin (43 ma), and the 30 kDa-ABP were common to both fractions. The species at 200 kDa observed in cytoskeletons (lane 2) likely was a degradation product of myosin II heavy chain (determined by immunoblotting; data not shown) that occurred despite the inclusion of protease inhibitors.

In addition to sharing cytoskeletal proteins, EP and cytoskeletons contained four Con A-binding proteins (gp152, gp130, gp105, gp70; Figure 3B) that corresponded to glycoproteins apparently shared by EP and PM (see Figure 1B). Judging by their relative Con A-binding signals, the levels of gp152, gp130 and gpl00 were relatively higher in EP (lane 3) than in cytoskeletons (lane 4). In contrast, gp70 was a major Con A-binding protein of cytoskeletons (lane 4) that had a relatively weak signal in both EP (lane 3) and PM (see Figure lB, lane 3).

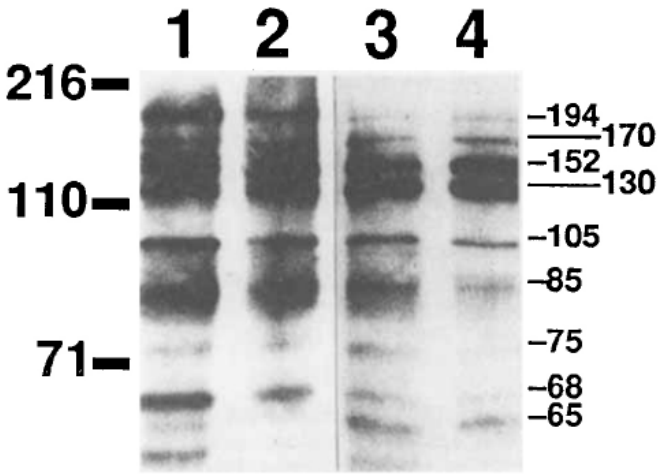

Figure 2. Integral membrane proteins of the PM were in EP. Blot (15 mg protein/lane) probed with biotin-Con A. PM: lane 1; NaOH-treated PM: lanes 2; EP: lanes 3; NaOH-treated EP: lanes 4. Molecular mass standards (kDa) are indicated to the left and labels to the right (offset for clarity) indicate co-migrating integral membrane glycoproteins. Only the 55-220 kDa region is shown.

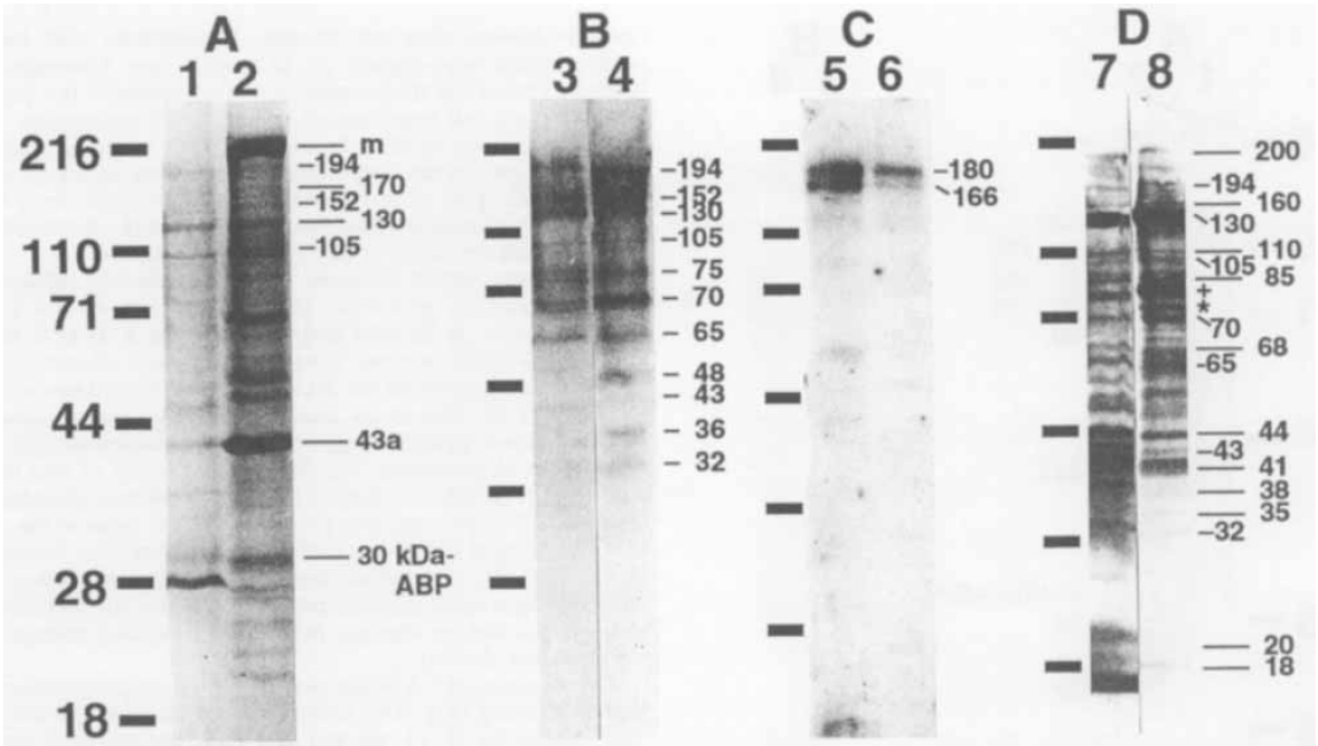

Figure 1. EP contained PM proteins. **A.** Coomassie Blue-stained gel (60 mg protein/lane). **B.** Autoradiogram of blot probed with ^{125}I-Con A. **C.** Autoradiogram of blot probed with ^{125}I -WGA. **D.** Surface-labeled proteins (detection by ECL). **B–D.** 40 mg protein/lane. PM: lanes 1, 3, *5* and 7; EP: lanes 2, 4, 6 and 8. Co-migrating protein species in PM and EP are labeled to the right of each panel and molecular mass standards (kDa) are indicated to the left of each panel. In **A**, myosin II heavy chain (m), actin (43a). and a 30 kDa-actin-bundling protein (30 kDa-ABP) are indicated. In **D**, a false positive detected in non-biotinylated samples is marked (+). Also indicated (*) is a species at 73 kDa detected in EP but not PM. Species labeled immediately to the right of **A** and **D** co-migrated with glycoproteins, whereas those labeled to the far right did not correspond to lectin-binding species.

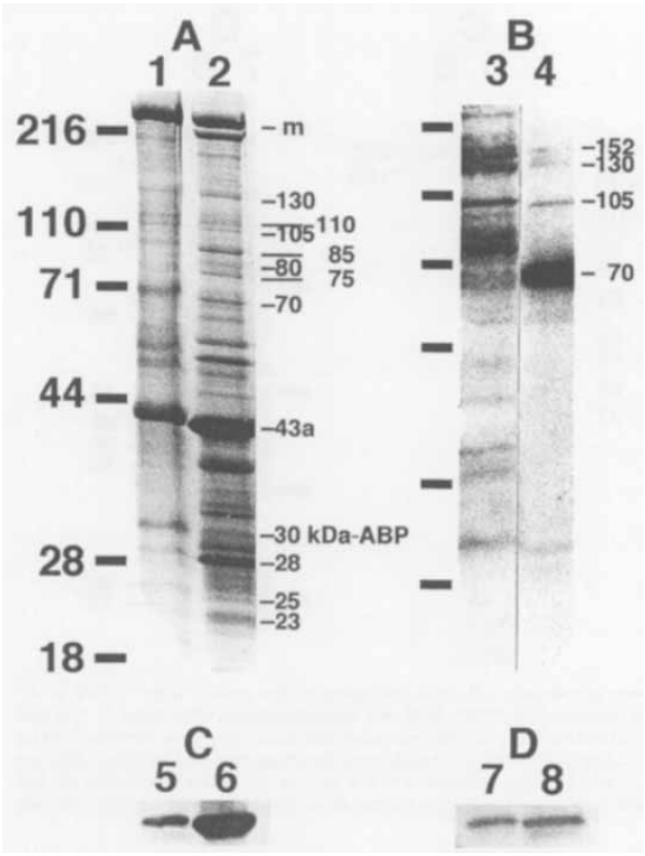

Figure 3. EP contained proteins found in cytoskeletons. **A.** Coomassie Blue-stained gel (60 mg protein/lane). **B.** Autoradiogram of blot probed with ^{125}I-Con **A** (40 mg proteidlane). **C.** Anti-actin immunoblot. **D.** Anti-30 kDa actin-bundling protein immunoblot. **C** and **D.** 20 mg protein/lane. EP: lanes 1, 3, 5 and 7; Cytoskeletons: lanes 2, 4, 6 and 8. In **A** and **B,** molecular mass standards (kDa) are indicated on the left, and molecular masses of co-migrating protein species found in EP and cytoskeletons are indicated to the right. Labels are offset for clarity.

The presence of actin (Figure 3C), the 30 kDa-ABP (Figure 3D) and myosin II (heavy chain; data not shown) in EP was confirmed by immunoblotting. By densitometry (average of three experiments), there was twofold more actin and 1.6-fold more of the 30 kDa-ABP in cytoskeletons than in EP when fractions were compared on an equal protein basis.

Changes in phagosomes as they matured. As phagosomes in macrophages mature, hydrolytic enzymes and proteins that mediate vesicle fusion are added through interactions with endosomal vesicles [11, 12, 42] while other phagosomal molecules are removed [1] or recycled to the surface of the cell [24, 36]. Similarly, we observed changes in the protein profiles of *D. discoideum* phagosomes over time.

Phagosomes collected 30 and 60 min after ingestion of beads contained less cytoskeletal protein than EP (Figure 4A). Densitometry of Coomassie-stained SDS-gels showed that there was 80% less myosin II heavy chain and 50% less actin in 30-min phagosomes, compared to EP. Densitometric scans of immunoblots confirmed a 60% reduction of actin (data not shown). Levels of the 30 kDa-ABP remained constant (verified also by immunoblotting; data not shown). Experiments with exogenously added myc-tagged 30 kDa-ABP (see Materials and Methods) ruled out the possibility that rebinding of this protein released upon cell lysis created an artefactual association. Species at 68, 65 and 41 kDa, detected at low levels in EP, seemed to increase over time. Since comparisons were based on equal protein loads, part of this apparent elevation might have been due to their increased contribution to the sample that occurred with the concomitant loss of cytoskeletal proteins.

There were varied increases in Con A-binding signals between EP and 30- or 60-min phagosomes (Figure 4B). A signal from a species at 70 kDa doubled, whereas a 43-kDa signal showed a fivefold increase from EP to 30- and 60-min phagosomes. Other species (at 85, 50, 41, 40, and 35 kDa) had modest increases of 10–20% in 30- and 60-min phagosomes relative to EP**.** Antibodies specific for gp130, a PM glycoprotein that may function in phagocytosis [6], showed that levels of this molecule were relatively unchanged from EP to 60-min phagosomes (Figure 4C). This glycoprotein probably was the same as the 130-kDa species that displayed a relatively constant Con A-binding signal (Figure 4B). In contrast, there were no Con A-binding proteins that decreased significantly from EP to 30- and 60-min phagosomes and no changes in the WGA-binding protein profile (data not shown).

The vacuolar H^+-ATPase was identified in phagosomes by immunoblotting (Figure 5A). Cells were fed beads for 10 min and then chased for 0, 15, 60 and 300 min. We prepared phagosomes using an alternative method (see Materials and Methods) in an effort to prevent release of peripheral proteins like the 70-kDa proton pump subunit. Prepared phagosomes were subjected to SDS-PAGE and western blot analysis using antibodies to the 100-, 70-, and 41-kDa proton pump subunits [15, 49]. As indicated in Figure 5A, all three subunits were substantially enriched in 10-min phagosomes relative to their levels in the PNS, which was used as the background control because this extract contains lysosomes and the contractile vacuole network where the proton pump ATPase has been localized [32, 33]. Due to its loss during phagosome preparations, the signal for the 70-kDa subunit was weaker than the signals for the other subunits. The relative amounts of the three subunits in phagosomes appeared relatively constant during the chase.

The immunoblotting findings indicated that the complete vacuolar proton pump was present in phagosomes perhaps as early as 10 min after beads were fed to cells. To determine if phagosomes were acidic (implying the proton pump was active) cells were fed FITC-labeled bacteria for 1 h., washed and then resuspended in fresh growth medium in the presence of 3 μm acridine orange. This chemical accumulates in acidic compartments where it fluoresces red-orange. As seen in Figure 5B, > 90% of the FITC-containing phagosomes also contained sufficient acridine orange that fluoresced red-orange (Figure 5C). Similar results were also observed when beads were fed to cells (data not shown). The acidification of phagosomes was consistent with the increased levels of the ATPase as vesicles progressed through the phagocytic pathway.

Discussion

Phagosomes from *D. discoideum* were examined in order to establish that these vesicles contained molecules from both the PM and the actin-based cytoskeleton. Enzyme assays verified that latex bead-containing phagosomes isolated on sucrose gradients were relatively enriched in lysosomal acid phosphatase and minimally contaminated with other membranes (Table 1). We also observed that membranes collected in significant amounts at the 10–25% sucrose interface on step gradients only when latex beads were fed to cells, indicating the purity

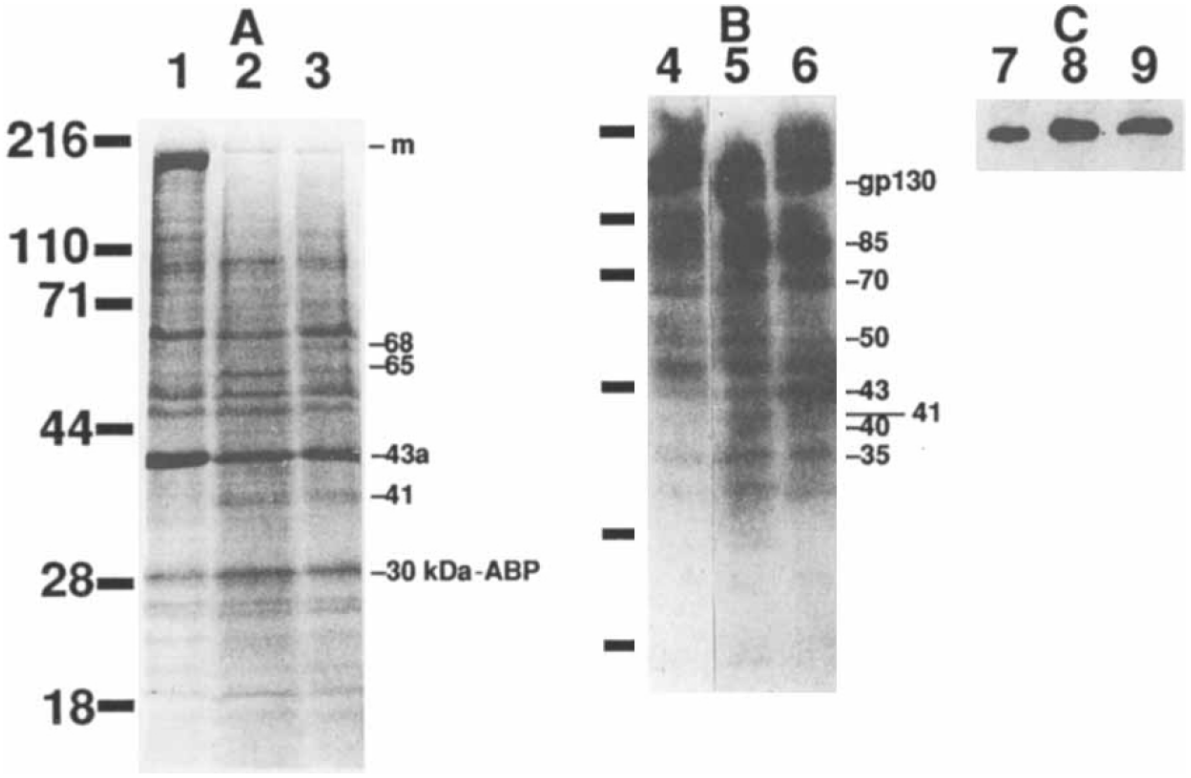

Figure 4. Changes in protein profiles during phagosome processing. **A.** Coomassie Blue-stained gel (60 μg proteinflane). **B.** autoradiogram of ^{125}I Con A-binding proteins (40 μg proteidane). **C.** Anti-gp130 immunoblot (20 pg proteidlane). EP: lanes 1, 4 and 7; 30-min phagosomes: lanes 2, ***5*** and 8; 60-min phagosomes: lanes 3, 6 and 9. In **A,** myosin II heavy chain (m), actin (a), and a 30 kDa actin-bundling protein (30 kDa-ABP) are indicated. Molecular mass standards (kDa) are indicated to the left of **A** and **B.** Signals that changed in intensities are marked to the right of each panel.

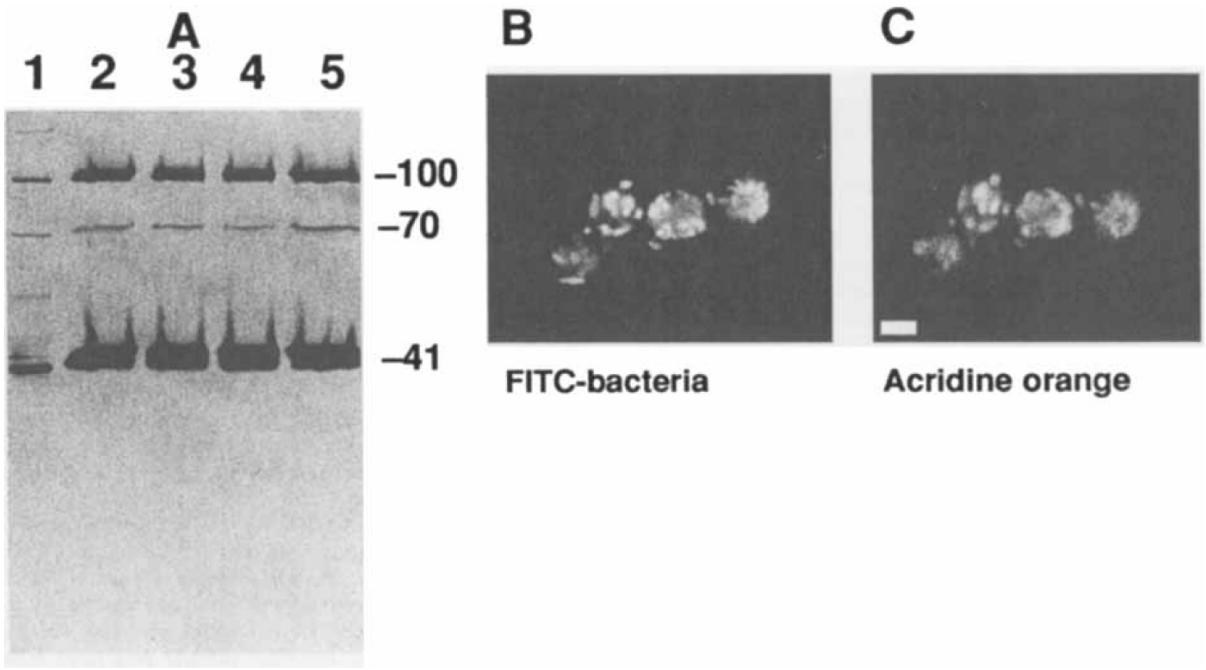

Figure 5. Phagosomes contained the vacuolar H^+-ATPase and were acidified. **A.** Protein blot (10 μg protein/lane) probed with antibodies to the 100, 70, and 41 kDa subunits of the vacuolar H^+-ATPase. PNS: lane 1; EP: lane 2; 30-, 60- and 300-min phagosomes: lanes 3,4 and 5, respectively. **B.** FITC-labeled bacteria ingested by *D. discoideum* cells. **C.** acridine orange fluorescence coincided with the bacteria of the cells in B. Bar = 5 μm.

of our preparations. Comparative protein analyses verified that a number of EP proteins were derived from the PM (Figs. 1, 2) and a population of cytoskeletal proteins appeared to have relatively long-lived associations (60 min) with phagosomes (Figure 3).

The zipper model of phagocytosis [23] proposes that particle ingestion requires the interaction of cell-surface receptors with the entire particle surface and that the phagosome membrane remains closely adhered to the ingested particle. If the mechanism of phagocytosis in *D. discoideum* proceeded according to the zipper model, we might expect an enrichment of receptors in newly formed phagosomes. The idea that specific receptors involved in mediating phagocytosis are included in nascent phagosomes is supported by the selective removal of Fc-receptors from mouse macrophage PM during phagocytosis [32] and by the concentration of complement receptor in phagocytic vacuoles of human monocytes containing *Legionella pneumophila* [8]. Generally, however, we found that the relative levels of Con A-binding and surface (biotin)-labeled signals within EP were comparable to those of PM (Figure 1), indicating that phagosomes largely contained a representative sampling of PM proteins. This finding was similar to the analyses by Cohn and co-workers [24, 34, 35] who found that phagolysosomes (containing latex beads) from macrophages and fibroblasts had the same relative proportion of cell-surface proteins as the PM of those cells.

We did observe that several PM proteins appeared to be excluded or partially excluded from phagosomes. A WGA-binding protein, gp166, had a consistently weaker signal in phagosomes relative to PM (Figure 1C) and from the surface (biotin)-labeled protein profile, there was an absence from EP of a prominent 17 kDa PM species (Figure 1D). Clemens and Horwitz [8] observed the exclusion of class I and II major histocompatibility complex molecules from monocyte phagosomes containing either *E. coli* or *L. pneumophila,* and suggested that PM proteins irrelevant to phagocytosis may be excluded during phagosome formation. Alternatively, the depletion of species from *D. discoideum* EP could be explained by their rapid return to the PM. Rapid recycling is possible based on studies of *D. discoideum* pinocytosis that showed that turnover equivalent to the PM surface area occurs every 45 min [51]. Muller et al. [36] showed that the recycling of macrophage membrane components from phagosomes back to the PM also was rapid and on the order of minutes. Finally, because the phagosomes were being acidified (Figure 5), it is possible that degradation or a modification of the proteins could have masked their detection.

The association of cytoskeletal proteins in phagosomes (Figs. 3A, 3C–D, 4A) was predicted from biochemical and microscopy studies showing that myosin, actin and actin-binding proteins are involved in macrophage [1, 12, 47, 48] and *D. discoideum* phagocytosis [16, 17, 58]. Reagents disrupting filamentous actin, such as cytochalasin A, inhibit *D. discoideum* phagocytosis [29], supporting the involvement of the cytoskeleton in this process.

The three monitored cytoskeletal proteins varied in their degree of dissociation from phagosomes over time. There were noticeable reductions in actin and myosin levels from EP to 30-min phagosomes (50% and 80%, respectively; Figure 4A and immunoblotting analyses). The loss of actin over time similarly was observed by immunofluorescence microscopy of zymosan particles ingested by macrophages [1]. The initial high level of myosin, which is believed to have a nonessential role in phagocytosis [45], and its subsequent reduction may be attributed to its interaction with actin. In contrast, levels of the 30 ma-ABP remained constant in EP, 30- and 60-min phagosomes. It was shown, however, by microscopy that the 30 kDa-ABP was enhanced in phagocytic cups and subsequently depleted from phagolysosomes [16, 17]. Re-binding of this protein to our phagosome preparations was minimal because added myc-tagged 30 kDa-ABP did not attach to vesicles in whole cell lysates. A possible explanation for the even levels of the 30 kDa-ABP is that there was a relatively rapid and immediate dissociation of the protein from phagosomes, and the signal observed on immunoblots was from residual protein. Also, because we lack an unchanging internal marker that would allow normalization of samples (for example, on a per phagosome basis), our comparative analyses of EP, 30- and 60-min phagosomes were based on a equal protein loads. The large amounts of actin and myosin in EP meant that the other cytoskeletal and membrane proteins were under-represented. Thus the level of the 30 kDa-ABP likely was higher in EP than in 30- and 60-min samples.

The cytoskeletal fraction also contained PM proteins (Figure 3B), as observed by others [40], and has been suggested to contain receptors that may associate with the cytoskeleton [39, 52]. Four of the nine integral membrane proteins of EP that bound Con A (gp65, gp105, gp130 and gp152; Figure 2) co-migrated with Con A-binding proteins in cytoskeletons (Figure 3). We suggest that PM proteins found also in cytoskeletons and EP to be potential links between the PM and the cytoskeleton. The best candidate appeared to be gp105 because its Con A-binding signal was relatively strong in both EP and cytoskeletons. Because both gp130 and gp152 had weak signals in cytoskeletons, they either did not have a genuine interaction with the cytoskeleton or the detergent-extraction procedure used to prepare cytoskeletons disrupted the interaction. Surface-labeling experiments support the idea that there is a 70-kDa PM glycoprotein (data not shown) so the presence of a Con A-binding protein at 70 kDa in cytoskeletons suggests its enrichment in this fraction. Whether this interaction occurs in vivo is under further study.

Except for changes in actin and myosin levels, few changes were observed in Coomassie Blue-stained gels of EP, 30- and 60-min phagosomes (Figure 4A). Blots probed with Con A, however, showed that some modifications of phagosomes had occurred. Con A-binding proteins at 85, 70, and 43 kDa were enhanced in 30- and 60-min phagosomes compared to EP (Figure 4B). Signals at 41, 40 and 35 kDa also appeared slightly stronger with time. The absence of significant changes in maturing phagosomes of macrophages as monitored by SDS-PAGE was noted by Pitt et al. [41]. However, protein blots probed with specific antibodies for endosomal-associated proteins showed differences during phagosome processing. Muller et al. [36] saw an enrichment in mouse macrophage phago-lysosomes of a 21 kDa PM protein using immunoprecipitation with a monoclonal antibody. Mellman et al. [32] was able to follow the fate of the Fc receptor also with an antibody. The introduction of the vacuolar H^+-ATPase to *D. discoideum* phagosomes was detected by immunoblotting (Figure 5; [37]). Thus, changes in protein levels during phagosome processing may not be discerned when examining the proteins as a group, as in SDS-gels, and may require specific reagents to distinguish differences in quantities and organization. Similarly, examination of specific *D. discoideurn* proteins may reveal subtle changes in protein quantities and organization unnoticed in total protein comparisons.

Another possible explanation for the lack of major detectable changes in the protein profiles of phagosomes is that indigestibility of latex beads prevents normal processing, as suggested by Chastellier et al. [10]. In this study, phagosomes containing *Mycobacterium avium, Bacillus subtilis* and latex beads were compared. Macrophage phagosomes containing undegraded latex beads do not mature into phagolysosomes. They remain fusogenic with early endosomes and do not acquire digestive enzymes as quickly as phagosomes containing bacteria. Since phagosome maturation and delivery of lysosomal enzymes involve major events of membrane recycling that presumably require free membrane exchange [10, 50], the tightly wrapped membranes around latex beads appear to hinder this process. *Dictyostelium* cells are incapable of digesting latex beads, and it is plausible that phagosome matura-

tion was retarded, as in macrophages fed latex beads. The unchanging levels of gp130 (Figure 4C), a postulated phagocytosis receptor [6] that was expected to be removed from vesicles, may have been the consequence of indigestible beads that arrested normal membrane recycling.

While it is possible that latex beads interrupted the conversion of phagosomes into phagolysosomes, acidification, presumably by the vacuolar H+-ATPase, did occur rapidly (Figure 5). This finding agrees with immunocytochemical [37] and biochemical [38] studies of *D. discoideum* that showed a proton pump to be present in vacuoles corresponding to acidosomes, the contractile vacuole complex, lysosomes and phagosomes but not on plasma membranes [35, 55]. Phagosomes isolated 2 min after cells were exposed to beads showed no enrichment of the proton pump (unpublished observations) supporting the idea that pump, absent from the plasma membrane, is then delivered to the phagosome. Acidification of phagosomes in macrophages also occurs quickly and is mediated by a vacuolar H^+- ATPase [3, 28]. Bouvier et al. [3] suggested that acidification and phagosome-lysosome fusion are regulated by different mechanisms, which is consistent with our observation that acidification of latex bead-containing phagosomes was not coupled to phagosome maturation.

As expected, both PM and cytoskeletal proteins were found within nascent phagosomes. As latex bead uptake is likely nonspecific [9], the inability to detect an enrichment of specific receptors in this study was not a surprise. Bacteria or bacteria attached to latex beads (which would allow phagosome collection based on their buoyancy) would be more suitable 'ligands' for the identification, and potentially, the isolation of specific phagocytosis receptor(s) of *D. discoideum*. EP, however, contained molecules presumably involved in mediating membrane-cytoskeletal interactions. EP thus should be useful in identifying specific molecular associations through, for example, crosslinking studies, which should provide insights into the mechanisms linking membrane receptors to the cytoskeleton during the process of phagocytosis.

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