Proteomics Fingerprinting of Phagosome Maturation and Evidence for the Role of a $\Gamma\alpha$ during Uptake

Daniel Gotthardt, a,b,c Vincent Blancheteau, c,d Mauro Delorenzi, e,f,g,h and Thierry Soldati i,j

Phagocytosis, whether of food particles in protozoa or bacteria and cell remnants in the metazoan immune system, is a conserved process. The particles are taken up into phagosomes, which then undergo complex remodeling of their components, called maturation. By using two-dimensional gel electrophoresis and mass spectrometry combined with genomic data, we identified 179 phagosomal proteins in the amoeba Dictyostelium, including components of signal transduction, membrane traffic, and the cytoskeleton. By carrying out this proteomics analysis over the course of maturation, we obtained time profiles for 1,388 spots and thus generated a dynamic record of phagosomal protein composition. Clustering of the time profiles revealed five clusters and 24 functional groups.

Institute for Medical Research, Department of Molecular Cell Research, Max Planck Institute for Medical Research, Department of Internal Medicine IV, University Hospital of Heidelberg, and Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), D-69120 Heidelberg, Germany, Department of Biological Sciences, Imperial College, London SW7 2AZ, United Kingdom, ISREC National Centre of Competence in Research (NCCR) Molecular Oncology, Swiss Institute of Experimental Cancer Research (ISREC), Epalinges, CH-1006 Switzerland, ISREC Swiss Institute of Bioinformatics (SIB), CH-1015 Lausanne, Switzerland, and Département de Biochimie, Faculté des Sciences, Université de Genève, CH-1211 Genève-4, Switzerland.

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The abbreviations used are: ER, endoplasmic reticulum; 2D, two-dimensional; NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble NSF attachment protein receptor; PMF, peptide mass fingerprint; HSP, heat shock protein.

Phagocytosis is the complex process by which eukaryotes ingest large particles of over 200-nm diameter. Many primitive eukaryotic cells phagocytose food particles, whereas in metazoans so-called “professional phagocytes” like the neutrophils and macrophages of the immune system specialize in engulfing invading microorganisms and parasites as well as necrotic and apoptotic cell remnants. Usually phagocytosis is triggered by contact of the particle with the cell surface, which induces its actin-mediated engulfment into a membrane-bound phagosome derived from the plasma membrane. Other endomembrane compartments such as endosomes and the endoplasmic reticulum (ER) contribute to the newly formed phagosomes. After killing of the engulfed microorganism, the contents of the phagosome are digested, and finally the lysosomal hydrolases are recycled for further use. During this maturation process, the phagosome remodels the protein and lipid components of its membrane as well as the soluble luminal components. Maturation of the phagosome is thus a normal part of its function, but the organelle can also be remodeled by some bacterial pathogens and eukaryotic parasites that “hijack” phagosomes and use them as a refuge where they can proliferate and evade the surveillance of the immune system.

Numerous studies have contributed to our understanding of the importance of many factors in phagosomal maturation including phosphoinositides (3) and other lipids (4), small GTPases (5), signaling and actin dynamics (6, 7), and fusion with endocytic compartments (8, 9). Yet despite a century of study, the mechanisms of phagocytic uptake and maturation are still relatively poorly understood. Exciting progress has been made, however, since the advent of large scale proteomics methods, which have revealed new facets of this organelle (10). By combining two-dimensional (2D) gel electrophoresis with mass spectrometry, the first proteomics analysis of phagosomes from mouse macrophages identified over 140 proteins (11). This analysis found diverse protein classes including not only the expected lysosomal proteins but also a large variety of proteins involved in regulating membrane trafficking, such as SNAREs and Rab GTPases as well as a subset of heterotrimeric $G$ protein subunits involved in signal transduction and many others (11). Despite these pioneering studies and recent technical advances, time-dependent organelle proteomics is still in its infancy. This is largely due to the paucity of appropriate bioinformatics tools to extract and integrate large scale and time-profiled proteomics data.
The social amoeba Dictyostelium is a very effective phagocyte, and its experimental versatility makes it an ideal candidate for multidisciplinary studies of cell function. Its genome is fully sequenced, assembled, and thoroughly and accurately annotated (12), confirming that amoebae are the closest group to metazoa and fungi. Large scale analyses are now possible in Dictyostelium by random insertion of plasmid sequences (13), microarrays (14), and proteomics (15, 16). Dictyostelium is also a well established model organism in which to study interactions between the host cell and a variety of human pathogens (17) including Legionella (18, 19), Mycobacterium (20), and Pseudomonas aeruginosa (21, 22). Furthermore the morphology and mechanisms of macrophagocytosis and phagocytosis in Dictyostelium are very similar to those in metazoa (16, 23–26), Protozoan amoebae in general are natural hosts for bacterial pathogens and can be made to host experimental species of bacteria (27). Proteomics studies of phagosomes isolated from the amoeba Entamoeba histolytica, for example, have revealed several aspects of phagosome signaling, uptake mechanisms, and time-dependent maturation in common with phagocytosis in mammalian professional phagocytes (28–31). Interestingly as in mouse phagosomes, a subset of heterotrimeric G protein subunits were identified in the phagosomes of E. histolytica (31).

Here we used a combination of time-resolved 2D gel electrophoresis and mass spectrometry-assisted protein identification to generate a protein history of the life of a phagosome in Dictyostelium, incorporating 179 phagosomal components. By clustering proteins that appear and disappear from the phagosome at similar times, we defined groups of proteins and functions that can be placed on a flow chart of phagosome maturation. Validating this approach, we found that two heterotrimeric G protein subunits, Gα5 and Gβ1, belong to two distinct but related groups of proteins present at early times in ablationes maturation. By studying Dictyostelium strains with ablationes encoding Gα5 and Gβ1 using a flow cytometry-based assay for phagocytic uptake, we demonstrated that both Gα5 and Gβ1 function in an early step of phagocytosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Dictyostelium discoideum cells of wild-type strain AX2 were grown axenically in HL5c medium (32) supplemented with 10 units/ml penicillin and 10 μg/ml streptomycin on plastic dishes or in shaking culture (at 180 rpm) at 22 °C. Mutant cell lines were selected and grown in HL5c medium supplemented with 5 μg/ml G418.

**Antibodies—**The antibodies used in this study were mouse monoclonal antibodies and rabbit polyclonal antibodies raised against Dictyostelium proteins as listed in Supplemental Table SII.

**Quantitative Immunoblotting—**After SDS-PAGE (33) and transfer onto nitrocellulose membranes (Protran, Schleicher & Schuell), immunodetection was performed as described previously using horseradish peroxidase-coupled goat anti-mouse or goat anti-rabbit IgGs (BioRad) at 1:5,000 dilution (34). Detection was performed with ECL Plus (Amersham Biosciences) using a chemiluminescence imager (LAS-1000, Fuji Film). Data quantification was carried out with Image Gauge version 3.0 (Fuji Film).

**Buffers—**Soerensen buffer contained 15 mm KH2PO4, 2 mm Na2HPO4, pH 6. Homogenization buffer contained 20 mm HEPES-KOH, pH 7.2, 0.25 μm sucrose, 1 x CompleteTM EDTA-free protease inhibitor (Roche Applied Science). Membrane buffer contained 20 mm HEPES-KOH, pH 7.2, 20 mm KCl, 2.5 mm MgCl2, 1 mm DTT, 20 mm NaCl. Storage buffer contained 25 mm HEPES-KOH, pH 7.2, 1.5 mm magnesium acetate, 1 mm NaHCO3, 1 μM CaCl2, 25 mm KCl, 1 mm ATP, 1 mm DTT, 1 x Complete EDTA-free protease inhibitor, 100 mm sucrose.

**Isolation of Phagosomes—**Phagosomes were prepared as described before (16, 35) and as briefly outlined in the supplemental information.

**Carbonate Extraction—**Carbonate extraction of phagosome membranes was carried out as described previously (36). In brief, freshly prepared phagosome pellets (about 7 x 1010 phagosomes prepared from 1.5 x 109 cells containing an average of 46.7 beads/cell) were resuspended in carbonate buffer (200 mm Na2CO3, Sigma, pH 11) by vortexing. After homogenization by five passages through the needle of a 1-ml insulin syringe, samples were kept on ice for 1 h. Stripped phagosome membranes were repelled by ultracentrifugation for 1 h at 100,000 x g in a Beckmann ML130 rotor. Pellets were resuspended in lysis buffer containing 7 mm urea (GE Healthcare), 2 mm thiourea (Sigma, pH 11) by vortexing. After homogenization by five passages through the needle of a 1-ml insulin syringe, samples were kept on ice for 1 h. Stripped phagosome membranes were repelled by ultracentrifugation for 1 h at 100,000 x g in a Beckmann ML130 rotor. Pellets were resuspended in lysis buffer containing 7 mm urea (GE Healthcare), 2 mm thiourea (Amersham Biosciences), 2% (w/v) CHAPS (Calbiochem), 2% (w/v) ASB-C80 (Calbiochem), 1% (w/v) DTT (Pharmacia Biotech), 2% (w/v) ampholytes (IPG buffer, pH 3–10 non-linear, Amersham Biosciences), and a protease inhibitor mixture (Complete EDTA-free). After sonication, isoelectric focusing gel electrophoresis was carried out as described below. Supplemental information is available.

**Sample Preparation and 2D Gel Electrophoresis—**Intact purified phagosomes were resuspended in lysis buffer containing 7 mm urea (Merck), 2 mm thiourea (Merck), 2% (w/v) CHAPS (Sigma, 1% (w/v) DTT (Sigma), 2% (w/v) Ampholite (IPG buffer, pH 3–10 non-linear, Amersham Biosciences), and a protease inhibitor mixture (Complete EDTA-free, Roche Applied Science) (37, 38). Suspensions were sonicated 3 x 10 min at 4 °C in a bath sonicator, incubated at room temperature for 2 h, and centrifuged for 60 min at 75,000 x g in a Beckmann TL120 centrifuge, and supernatants were stored at −80 °C until further use.

Extracts were separated in the first dimension using 18-cm strips with immobilized non-linear gradients from pH 3–10 (pH 3–10) (37, 39–42) with minor modifications described in the supplemental information. 2D gels were either stained with silver (43) or with Coomassie Blue (Novex/Invitrogen) according to the manufacturer’s instructions. The gels were scanned using a Sharp JX-330 scanner and ImageMaster Labscan software. Procedures were carried out under standardized conditions for all gels. Supplemental information is available.

**Mass Spectrometry—**Individual spots were excised from 2D gels, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin as described previously (44). Following digestion, tryptic peptides were extracted from the gel pieces with 50% acetonitrile, 0.1% TFA; concentrated; and analyzed by mass spectrometry.

For peptide fingerprinting by MALDI-TOF mass spectrometry (Ultraflex, Bruker), samples were desalted using ZipTip (Millipore) according to the manufacturer’s instructions and spotted onto a steel target using α-cyano-4-hydroxycinnamic acid as matrix. The peptide mass fingerprint (PMF) was acquired after external calibration (peptide calibration standard II, Bruker) in positive ion reflector mode. For protein identification by PMF, peptide masses were labeled manually using the SNAP algorithm (signal to noise ratio = 3; quality factor threshold, 100) (flexAnalysis, Bruker) by comparison with a control sample taken from a spot of an empty area of the same gel. The PMF
was searched against the Dictyostelium database (protein sequences for dictyBase primary features, 13,676 sequences, at dictybase.org) using Mascot version 2.0.5 (Matrix Science). The algorithm was set to use trypsin as the enzyme, allowing for one missed cleavage site and assuming carbamidomethyl as a fixed modification of cysteine and oxidized methionine as a variable modification. Mass tolerance was set to 100 ppm unless otherwise indicated. Protein hits were considered identified if the Mascot score exceeded the significance level ($p > 0.05$).

For peptide sequencing by ESI Q-TOF mass spectrometry, peptides were desalted and concentrated using custom-made chromatographic columns (Poros 50 R2, Perseptive Biosystems) (45). They were eluted directly into a precocated borosilicate nanoelectrospray needle (MDS Protana, Odense, Denmark). Mass spectrometry was performed on a Q-TOF mass spectrometer (PE Sciex, Weiterstadt, Germany) equipped with a nano-ESI ion source (MDS Protana). A potential of 900 V was applied to the nanoelectrospray needle. De-clustering potential and focusing potential were set to 40 and 100, respectively. Fragmentation of selected peptides (unit resolution) was usually performed at three different collision energies (22, 27, and 55 V). The data were processed using the Bioanalyst software (PE Sciex).

**Image Processing and Dataset and Statistical Analyses—**The digitalized 2D gels of the time series were analyzed using the Phoretix 2D Evolution (version 2005) software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) for spot detection, gel matching, and background correction (mode of nonspot, vector size 100 pixel), and these data were normalized to the sum of the total spot volume. Data were exported to a spreadsheet program (Microsoft Excel).

**Temporal Profile Data Analysis—**Data analysis was performed in R (cran.r-project.org). For data normalization, the spot intensity vectors per time point were first scaled to a constant sum of intensities, and then the time series vector per spot was rescaled so that the maximum intensity was standardized to a value of 1,000. Spots were classified into groups with similar time profiles with the partitioning around medoids (PAM) algorithm (46) and a predefined number of 24 groups because silhouette width values did not support any specific number of groups in the data. Details about the choice of this value of 24 is available in the supplemental information. Only the 898 spots that were found in at least two time points were used. Of the 490 spots detected at only one time point, only one representative from each of the six time points was taken for analysis. The distance measure was $1 −$ Pearson correlation for the transformed variable $Z = \log_2(1 + \text{ratio of the intensity to the mean intensity})$. Color-coded intensity plots for $Z$ were produced with the function "heatmap." Relationships between the 24 groups were visualized in a hierarchical clustering dendrogram of their average profiles (hclust function in R, average linkage method, $1 −$ Pearson correlation as distance). For cross-correlation analysis, the pairwise Pearson correlations were computed between each of the averaged profiles of the 24 groups, and this correlation matrix was represented on a false color scale.

**Flow Cytometry-based Uptake Assay—**We used a flow cytometry-based particle uptake assay detailed in the supplemental information. Briefly $10^7$ cells grown on plates were harvested, resuspended in 5 ml of HL5c medium, placed in one well of a 24-well plate, and shaken at 150 rpm. After washing, $2 \times 10^6 1-\mu m$ fluorescent beads (Fluoresbrite YG 1-μm microspheres, Polysciences, Inc.) were added to be cells in suspension and incubated at room temperature under constant shaking at 120 rpm. At each time point, 0.5 ml of cells was harvested, and bead uptake was stopped. Then cells were centrifuged at $500 \times g$ for 5 min at 4°C, resuspended in 0.5 ml of ice-cold Soerensen buffer containing 120 mM sorbitol, and kept on ice until fluorescence-actuated cell sorter data acquisition. For each time point, 30,000 fluorescence events were acquired using a FACScan flow cytometer (BD Biosciences), and bead uptake was quantified. Mean fluorescence was calculated by analyzing histograms showing fluorescence versus events. Supplemental information is available.

**RESULTS AND DISCUSSION**

In analyzing the proteome of Dictyostelium phagosomes, we had two aims: to identify new protein components and to see how phagosome proteins change during maturation. To do so, we prepared large amounts of latex bead phagosomes by using a protocol that includes physiological concentrations of ATP, ensuring high yields and purity (see supplemental information). Briefly using markers for a variety of organelles, we calculated that our phagosome fractions were enriched up to about 100-fold over a crude membrane fraction and were depleted about 10-fold of major contaminants, such as contractile vacuole, endoplasmic reticulum, and mitochondria (16, 35). In addition, our pulse-chase protocol allows purification of phagosome at various times that were shown previously to cover all the steps of Dictyostelium phagosome maturation from uptake through digestion and finally to exocytosis of the undigested remnants (16). We separated phagosome proteins by 2D gel electrophoresis using preparative gels from which we picked spots and identified the corresponding proteins (Fig. 1) and analytical gels of six different time points during maturation from which we computed a time profile for each spot (Figs. 2 and 3).

**Identification of Phagosome Proteins—**We began by applying standard protein extraction, solubilization, and 2D gel procedures (see “Experimental Procedures”) to analyze the prepared phagosomes. This resulted in highly reproducible gels with very good spot quality (see for example, Fig. 1A (gel A); see also Ref. 16). However, because these procedures do not solubilize hydrophobic and transmembrane proteins very well, we also explored a variety of the latest generation detergents (see supplemental information) as well as various extraction methods and 2D gel protocols. Carbonate extraction (36) followed by solubilization in a buffer containing 2% CHAPS and 2% 4-octylbenzylamidopropyl(dimethylammonio)-propanesulfonate (47) yielded excellent results; a preparative gel (gel B) from a phagosome extract prepared according to this method is shown in Fig. 1B.

A total of 180 and 232 spots were picked from gels A and B, respectively, and analyzed by peptide fingerprinting (see “Experimental Procedures” and Supplemental Figs. S1 and S2 for spectra). Among these, 11 spots were keratin, and 72 others could not be identified for technical reasons. In addition, four proteins corresponding to spots 134 and 135 of gel A and spots 136 and 166 of gel B were identified by ESI-MS/MS (see Supplemental Table S1 and Figs. S1 and S2 for representative spectra). In total, 137 spots from gel A and 192 spots from gel B were identified as known proteins or predicted open reading frames in the Dictyostelium genome (dictybase.org (12)). Taking into account the proteins common to both gels and counting proteins present as multiple spots, including degradation products, as one protein, a total of 179 different proteins were finally identified. Table I lists all these...
proteins, their location(s) on each gel, their known or proposed functions and cellular locations, whether they were found previously in the mouse phagosome proteome (11), and whether their presence has been confirmed by our ongoing large scale analysis of Dictyostelium phagosomes by one-dimensional gel electrophoresis and liquid chromatography tandem mass spectrometry. Supplemental Table SI presents additional basic information about each spot identification.

The phagosomal proteins we identified belonged to a variety of functional classes (Fig. 1C). Reflecting the digestive character of phagosomes and their export of nutrients to the cytosol, 37% of the proteins were members of the “energy, transport, and metabolism” class. The second largest group, comprising 13% of polypeptides, was proteins involved in membrane traffic, reflecting the importance of transport to and from the maturing phagosome. Among them, we found a large collection of small GTPases, some of which are known

![2D gel electrophoresis of phagosome proteins, their predicted functions, and subcellular locations.](image)

Preparative 2D gels of phagosome proteins extracted under standard conditions (A) or under conditions that favor extraction of membrane proteins (B) are shown. The phagosomes analyzed in gel A are a pool of the early three time points of a pulse-chase feeding (see Fig. 2), whereas gel B is derived from a pool from the three late time points. The pH range of the first dimension isoelectric focusing gel is shown at the bottom, and the sizes of molecular mass standards for the second dimension are indicated on the left in kDa. The proteins corresponding to 137 spots on gel A and 192 spots on gel B (circled in blue) were identified by mass spectrometry and comparison with the Dictyostelium genome. Further data are compiled in Table I and Supplemental Table SI. The identified phagosomal proteins were classified according to their predicted or known functions (C) and subcellular locations (D).

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2 M. Desjardins, M. Hagedorn, R. Dieckmann, and T. Soldati, unpublished data.
to play a role in uptake processes in Dictyostelium (24, 48–50) and macrophages (51, 52). Of these, Rab11B, Rab14, Rab7, and Rap1 were also found in mouse phagosomes (11), and Rab7 was found in E. histolytica phagosomes (31), indicating that the phagocytic mechanisms have been conserved through evolution. In the membrane traffic group, we also detected members of the SNARE machinery responsible for the specificity of membrane fusion. Of these, only H9251- and H9253-SNAP (soluble NSF attachment protein) have been found in mouse phagosomes. We also found dynamin, a protein involved in vesicle fission.

Other large groups of proteins we identified function in signal transduction (9%) and cytoskeleton organization (7%). These signaling proteins included the heterotrimeric G protein subunits Gβ, another Gβ-like protein, and Gαζ, Gα2, Gβ1, and Gβ2, have been found previously in mouse phagosomes (11). Not surprisingly, in view of their richly documented role in phagocytosis, we found a large variety of cytoskeleton proteins, some of which have also been found in mouse phagosomes. In addition to the signaling and cytoskeleton functional groups, we found a large group of heat shock proteins (HSPs) and other chaperones (9% of all proteins). HSPs act as chaperones and assist protein synthesis, folding, and breakdown. They also regulate actin polymerization/capping, uncoating of clathrin-coated vesicles, and phagocytosis in macrophages (53). Besides proteins known to play a role in phagocytosis and known proteins for which a role in phagocytosis has not been described, the functions of over 35 conserved proteins we identified (8%) are not known. Additional research will be necessary to assess the role of these novel candidates in phagocytic functions.

Fig. 1D shows the known or predicted subcellular locations of the phagosome components we identified. Cytosolic proteins comprise 34%, endosomal and lysosomal proteins comprise 12%, and plasma membrane proteins comprise 8% of the total. The subcellular locations of 28% of them, however, are not known. This broad variety of functions and subcellular locations reflects the complex processes involved in the uptake of particles and the subsequent maturation of the phagosome into a killing and digestive compartment. It is impor-
tant to note that it is difficult to judge a priori the specificity of the presence of cytosolic proteins on phagosomes. As detailed in the supplemental information, some abundant cytosolic proteins are represented, but many are not. In addition, the fact that the presence of most proteins follows a complex temporal profile speaks against a simple piggy-backing during purification but in favor of a specific and regulated recruitment.

Many of the proteins we identified have evident roles to play at one or another stage of phagosome maturation (see above), but the presence of others is more surprising and might reveal additional processes linked to the phagosome.
TABLE I
Proteins identified from preparative gels

| Protein Name | Biological Function | Molecular Function | Predicted Function | Group | CS/160/114 Found in source | Comments |
|--------------|-------------------|-------------------|-------------------|-------|--------------------------|---------|
| ... | ... | ... | ... | ... | ... | ... |

This table lists the 179 proteins identified after picking spots highlighted on gels A and B in Fig. 1.
One such example is the identification of proteins from the ER, Golgi apparatus, and peroxisomes among the phagosome proteins. The presence of four ribosomal components, four tRNA synthetases, the 54-kDa subunit of the signal recognition particle, and three translation elongation factors (which are actin-binding proteins), all factors involved in protein biosynthesis, may be explained either by protein synthesis taking place on the phagosome itself or substantial association/fusion of phagosomes with the ER (54). This latter hypothesis is supported by the presence of a subset of ER-resident proteins (calreticulin, protein-disulfide isomerase, and a homologue of the immunoglobulin Binding Protein BiP) and ER export/trafficking regulators (Sar1 and Rab1D) in the phagosomes.

Dynamics of the Phagosome Proteome—To generate temporal profiles of phagosome proteins during maturation, we made use of our established pulse-chase protocol (see supplemental information). For each maturation stage we quan-
Phagosome Protein Dynamics

**Statistics of detection of protein spots**

| Time point | Not detected | Detected | At peak | Percentage at peak | Specific for this time | Percentage specific for this time |
|------------|--------------|----------|---------|-------------------|------------------------|-------------------------------|
| 5'/0'      | 359          | 1,029    | 472     | 34.0              | 259                    | 18.6                          |
| 15'/0'     | 698          | 690      | 226     | 16.3              | 68                     | 4.9                           |
| 15'/15'    | 812          | 576      | 204     | 14.7              | 30                     | 2.2                           |
| 15'/45'    | 877          | 511      | 178     | 12.8              | 54                     | 3.9                           |
| 15'/105'   | 937          | 451      | 159     | 11.5              | 38                     | 2.7                           |
| 15'/165'   | 897          | 491      | 149     | 10.7              | 41                     | 3.0                           |
| Sum        |              |          | 1,388   | 100.0             | 490                    | 35.3                          |
| Average    | 763          | 625      | 231     | 16.7              |                        |                               |

**Changes between time points**

| Time points | Appearing | Disappearing | Staying | Percentage staying |
|-------------|-----------|--------------|---------|-------------------|
| 5'/0' vs. 15'/0' | 130       | 469          | 560     | 54.4              |
| 15'/0' vs. 15'/15' | 178       | 292          | 398     | 57.7              |
| 15'/15' vs. 15'/45' | 194       | 259          | 317     | 55.0              |
| 15'/45' vs. 15'/105' | 198       | 258          | 253     | 49.5              |
| 15'/105' vs. 15'/165' | 219       | 179          | 272     | 60.3              |
| Average     | 184       | 291          | 360     | 55.4              |

**Detection statistics**

| Number of detections | Number of spots | Percentage |
|----------------------|-----------------|------------|
| 1                    | 490             | 35.3       |
| 2                    | 280             | 20.2       |
| 3                    | 195             | 14.0       |
| 4                    | 164             | 11.8       |
| 5                    | 97              | 7.0        |
| 6                    | 162             | 11.7       |
| Sum                  | 1,388           | 100.0      |

This simple analysis revealed substantial remodeling during the different phases. For example, the 5-min pulse (Fig. 2A) had the greatest number of different spots (1,029); of these, 259 were specific for this time point (more than at any other time point) (Table II). This complexity at early times likely reflects the fact that early phagosomes contain both proteins derived from the plasma membrane and newly recruited phagosome-specific proteins (16). Not surprisingly, comparison of the second time point with the first revealed the disappearance of 469 spots and appearance of 130 others (Table III). This substantial remodeling likely includes sorting to recycle plasma membrane proteins back to the surface (55, 56). Overall the total number of spots detected decreased over time except at the last time point (Table II). Vast remodeling of the phagosomes during maturation was indicated by the fact that, on average, 184 spots appeared and 291 spots disappeared between any two consecutive time points (Table III). Again on average, 35% of the spots detected were present only at one time point, 20% were detected at only two time points, and 7% were detected at five time points. Nevertheless 12% of all spots were present at all six time points (Supplemental Fig. S1 and Table IV).

In summary, this analysis clearly indicates distinct phases in the maturation program. We detected the most remodeling at the beginning and end of the pathway and thus propose that uptake and exocytosis are the most complex “multidisciplinary” stages, requiring integration of signaling, membrane trafficking, and cytoskeleton reorganization. The more “specialized” intermediate stages, we suggest, are more uniquely devoted to successive membrane trafficking steps necessary for efficient accumulation of digestive enzymes and extraction and transport of nutrients and may thus require a simpler repertoire of proteins.

**Temporal Changes in Phagosome Components**—The complexity of the maturation process can be seen from Fig. 3. The reference gel (Fig. 3A) shows an overlay of the 1,388 spots detected in the whole time series superimposed on the gel of time point 5'/0'. Spots that were present at two or more time
points are circled in blue or red: the 125 spots circled in red correspond to proteins identified from the preparative gels (see Table I, column “Group”). The 490 spots present at only one time point are circled in green. Although these spots include proteins that potentially are the most stage-specific, they are also most prone to artifact (degradation and spots that could not be matched to other gels), therefore they were not all included in the cluster analysis; instead six spots representative of the proteins that appear only at one of each of the six time points were included. The spots circled in blue or red were analyzed further to monitor how their intensity changed over time. Spot intensities were quantified by densitometry, normalized for the complete temporal profile, and depicted as heat maps in which red corresponds to high intensity and green corresponds to low intensity (see “Experimental Procedures”). To extend the 2D gel data, we also analyzed the pulse-chased phagosome preparations for the presence of known endosomal and phagosomal proteins by using quantitative Western blotting (Fig. 3C). This approach also allowed us to compare the data obtained for a selection of seven proteins by both Western blotting and 2D gels (see below). Again signal intensities were quantified by densitometry, normalized for the temporal profile, and depicted as heat maps (Fig. 3D).

Altogether we obtained 925 profiles (for 898 spots present at more than one time point, plus the profiles of the six spots representative of the 490 stage-specific spots, plus the 21 profiles obtained by Western blotting) representing 5,550 individual intensity measurements that are presented in the heat map in Fig. 3B. These “temporal profiles” were submitted to cluster analysis, a method also used to group microarray “expression profiles” according to their degree of similarity. Exploratory analysis using a variety of clustering methods and distance metrics gave fairly robust results and similar clusters and revealed that the profiles do not fall into a well defined number of well separated groups. Therefore, the optimal number of clusters was determined so that it would result in (a) a relatively homogeneous number of profiles per group (Fig. 4A), (b) fairly distinct average group profiles (as judged by inspection and by cross-correlation (Fig. 4B), see below), and (c) rather homogeneous groups (as judged by the standard deviation from the average group profile, Fig. 5 and Supplemental Fig. S3). Finally we used the partition around medoids (PAM) algorithm (46) to classify the 925 profiles into 24 groups (a detailed argument is presented in the supplemental information and Supplemental Fig. S2). At a higher level in the dendrogram there are five major clusters (Fig. 4, I–V) that correspond roughly to different times of maximal abundance of each protein during the maturation program.

Many of the 24 groups had simple average profiles, consistent with the proteins they represent having a major function at one stage of phagocytosis and with a relatively simple, linear model of phagosome maturation (Fig. 4C). For example, groups 1–6 had a major sharp peak of red (high spot intensity) and could easily be aligned in such a sequence in association with groups that have a slightly broader peaks (groups 8, 10, 19, and 22). About a fifth of the groups (groups 7, 9, 12, 13, and 17) comprise proteins that appeared at two stages of maturation, including the first time point for four of them. At the other extreme, some groups (groups 11, 16, 18, 20, and 21) comprise proteins present at most time points, perhaps reflecting an unchanging feature of phagosomes identity or a recurrent function. Finally some groups (groups 14, 15, 23, and 24) comprise proteins that appeared and disappeared from the maturing phagosomes in more complex ways, reflecting either a periodic need for a specific function or the existence of parallel maturation pathways in which they act at different times.

From Temporal Profiling to Functional Grouping—Cluster analysis revealed that the 24 groups are organized into five major clusters that are apparent on the tree structure (Fig. 4A, clusters I–V) and are also visible on the cross-correlation map as square regions of “hot” colors aligned along the diagonal (Fig. 4B, boxes). The groups inside each cluster share a major peak at a common time point, and this reflects the fact that the clusters (from I to V) can be roughly aligned along an axis of maturation in order of appearance of that major peak. This finding is also consistent with a simple, linear model of phagosome maturation as illustrated in Fig. 4C, but more complex alternative pathways of maturation are discussed below.

A detailed description of clusters, groups, and proposed associated functions in maturation is presented in the supplemental information, and the composition of each group is presented in Supplemental Figs. S2-1 to S2-6. Briefly looking at cluster I (groups 1, 2, 8, and 11) and the earliest time point, the presence of group 1 proteins reflects the involvement of the actin cytoskeleton, membrane trafficking, and molecules that bridge the two functions to trigger and carry out uptake; group 11 includes many enzymes, probably reflecting the early establishment of the degradative phases of phagocytosis, and group 8 includes Gαx, suggesting a signaling function during uptake (see below). Cluster II (groups 3, 9, 14, and 18) comprises a diverse collection of proteins likely reflecting the metabolic role of the phagosome as well as other functions that were proposed recently (see below). Cluster III (groups 4, 7, 12, 16, and 20) reflects functions related to late endosomes and multivesicular body formation, including the necessary signaling, cytoskeleton, and membrane trafficking machinery. The proteins representative of cluster IV (from groups 5, 13, 15, 21, and 22) also reflect late endosomal/lysosomal characteristics featuring digestive components as well as the components of trafficking associated with recycling/exocytosis. Cluster V (groups 6, 17, 19, 23, and 24) completes this series, finishing the evolution started in cluster IV, with the presence of factors typical of late endosomes and recycling/exocytosis, but the prominent digestive character of cluster IV is missing.
FIG. 4. Clustering and cross-correlation analyses of temporal profiles and flow chart of phagosome maturation. A, heat map resulting from cluster analysis of 925 temporal profiles and showing the average intensities over the six time points of the 24 groups of proteins indicated on the left by a Roman numeral (Groups). The number of profiles in each group (Nr profiles) and their attributed name (Names) are indicated on the right. The tree on the left also illustrates the computed relatedness of the groups and their organization into five clusters (clusters I–V). We named each group according to one representative known protein. When no known and studied protein was present in the group, we
Cross-correlation Analysis Reveals Further Relatedness between Functional Groups—Although clustering is important to integrate the information and reveal order in large datasets, a well known and inherent feature of most clustering methods is that sometimes related profiles end up in relatively distant clusters/groups. Therefore, to extend and strengthen the clustering data, we calculated what we call cross-correlation, that is all the pairwise correlation coefficients between the average profiles across all 24 groups. This analysis quantifies and highlights the strength of the relationship between attributed a name based on the dictyBase identification number. Group 24 contained no identified protein and was thus named after spot 660, which has a representative profile. B, heat map matrix of pairwise cross-correlation between the groups indicated by the Arabic numerals along the top and left sides. Red indicates identity, and the “cooler colors” indicate less relatedness. Clusters are indicated by Roman numerals on the left and by boxes along the diagonal. Additional strong correlations are emphasized by circles, squares, and diamonds (see main text). C, the scheme in the central panel illustrates the various phases of phagosome maturation from uptake of particles (stars on the left) to the final egestion of undigested remnants (small triangles on the right). Arrows indicate pathways of membrane traffic and crucial processes accompanying the various phases of maturation. The upper panel indicates the timing of acidification and neutralization. This scheme is based on one published previously for macropinocytosis (69). The lower panel shows the heat maps of the average temporal profiles of the 24 groups indicated at the left, illustrating the stages of the maturation program at which these groups of proteins are present.
any two average temporal profiles of contiguous or non-
contiguous groups of the hierarchical clustering tree. On the
resulting heat map matrix (Fig. 4
B
), the index of correlation
between the average temporal profiles of two groups is indi-
cated at the intersection by a color-coded square. Red (on the
diagonal) indicates identity, and decreasing similarity is indi-
cated by colors that become closer to green.

The cross-correlation map reveals additional relatedness
between groups outside the major five clusters, visible as
isolated or small groups of hot squares off the diagonal (Fig.
4B, emphasized by circles, squares, and diamonds). For ex-
ample, groups 1, 8, 12, and 13 are highly related (Fig. 4B, cir-
cles). In addition to the proteins already mentioned above,
group 12 also contains signaling and membrane trafficking
proteins, and group 13 also contains some enzymes. Similarly
groups 10 and 11 from cluster I are closely related to groups
3, 9, and 18 from cluster II, and finally group 10 from cluster
I is related to groups 7 and 20 from cluster III. Overall it
appears that group 10 has a remarkable position, being a
close relative of many groups, both inside and outside its
cluster. Altogether these complex patterns of appearance
and disappearance of some protein groups during matura-
tion emphasize that the linear maturation program depicted in
Fig. 4C is an oversimplification and should be completed by
complex cross-talk between endocytic and phagocytic or-
ganelles and/or the existence of alternative parallel maturation
pathways (57).

We also focused on proteins for which we had both West-
ern blotting and spot quantification data. For example, the
profiles obtained for vacuolin by Western blotting (with an
antibody that recognizes both vacA and vacB (58)) and for the
vacA spot both fall into group 19, but the profile for vacB is in
group 15. These groups are in two different but related clus-
ters (IV and V) and also show a relatively strong cross-corre-
lated average profiles. Similarly the profile obtained for G
H9252 by Western blotting (group 1) and the

Fig. 6. Quantification of phagocytosis in two Dictyostelium G protein mutants. Time courses of the phagocytic uptake of 1-μm
fluorescent microspheres by various strains of Dictyostelium are shown. A, fluorescence uptake by G4-null cells (red curve) and wild-type cells
(blue curve) measured by flow cytometry. C, fluorescence uptake by Gβ-null cells (red curve) and Gβ+ cells (blue curve) (see main text for
details). Uptake was normalized to wild-type cells or Gβ+ cells, respectively. B and D show the proportion of cells that acquired one or more
beads (red and dark blue curves) and the proportion of cells that contained one bead only (yellow and light blue curves). The red and yellow
curves indicate data for the mutant cell lines, and the dark and light blue curves indicate data for the wild-type and Gβ+ cells. Uptake was
assayed in triplicate, and error bars represent standard deviations.
corresponding GpbA spot (group 13) are neither in the same group nor the same cluster (clusters I and IV, respectively) but show highest pairwise cross-correlation. These data demonstrate that the use of different methods to obtain the temporal profiles can result in some degree of discrepancy and thus show some of the limitations of our approach. Nevertheless the discrepancies are small and do not really affect our overall conclusions and the concept presented here.

Our analysis of time-dependent proteomics data has allowed us to establish a model of phagocytic mechanisms that will be useful for further functional analysis in Dictyostelium and other organisms. We next aimed to test a prediction of this model as a proof of principle for future investigations.

**Heterotrimeric G Protein Function in Early Phagocytosis**—
Many signaling pathways are activated when a ligand binds to its G protein-coupled receptor; this receptor-ligand binding activates the downstream heterotrimeric G protein (consisting of one α, one β, and one γ subunit) thus converting the extracellular signal into an intracellular response. Subunits of trimeric G proteins have been reported in phagosomes from mouse (11) and *E. histolytica* (31), and a study based on use of inhibitors and toxins has found evidence for a role during phagocytosis (59). The latter data are contradicted by a recent report that knock-down of multiple Gβ and Gγ in a macrophage line abolished G protein-coupled signaling without affecting phagocytosis (60). In *Dictyostelium*, there are 14 different Gα subunits, one Gβ and one Gβ-like subunit (12), and one Gγ subunit. G proteins are essential in this organism for chemotaxis, cell aggregation, and differentiation. In particular, Gα4 (61) and Gα4 (62, 63) are important for chemotaxis and differentiation, but no Gα has been shown to play a role in phagocytosis. Gβ, on the other hand, is required for chemotactic responses and multicellular development as well as phagocytosis (64–66).

On our 2D gels, there was a strong signal from Gα4 at the two earliest time points (Fig. 5A, lower panel) suggesting that it may be involved in phagocytic uptake. Likewise group 13 proteins, including Gβ (GpbA), were also present around the beginning of phagocytosis (Fig. 5B). Interestingly both Gα4 and Gβ also peaked at a later time point, perhaps indicating a dual role both during the early uptake phase and in a late maturation phase that might reflect the documented role of Gβ in actin reorganization (66) and the function of the actin cytoskeleton in both uptake and exocytosis (67). Because of its presence on early phagosomes, we wondered whether Gα4 might also play a role in uptake similar to the function reported for Gβ in phagocytosis (66) and thus might be one missing link upstream of Gβ linking the Gαβγ complex to an unknown receptor. We therefore compared phagocytosis in cells deficient in Gα4 subunit (Gα4-null cells) with wild-type cells (Ax2 cells) and, as a positive control, cells deficient in Gβ subunit (Gβ-null cells) with cells expressing a fully functional His-tagged form of the Gβ (Gβ+). To do so, we used a flow cytometry-based particle uptake assay to monitor the uptake kinetics of brightly fluorescent beads (see “Experimental Procedures”). The knock-out strains grew at rates indistinguishable from their respective controls, demonstrating their relative fitness. Both mutant cell lines had a pronounced defect in bead uptake (Fig. 6). During the 120-min course of the assay, the Gα4-null cell population showed an uptake rate of fluorescent beads 50% lower than that of the wild-type cells (Fig. 6A), and the rate of uptake in the Gβ-null cell population was reduced to 40% of that of the Gβ+ cells (Fig. 6C). All the cells in both mutant strains possessed some phagocytic activity and were able to ingest at least one particle at a rate only slightly slower than their respective controls (Fig. 6, B and D, red curves compared with dark blue curves). By contrast, the phagocytic rate in both mutant strains was markedly slower than in the controls when the proportion of cells containing only one bead was plotted against time. In the wild-type and Gβ+ strains, this population appeared and disappeared rapidly (Fig. 6, B and D, light blue curves), whereas in both mutant strains (yellow curves), the peak of cells with only one bead appeared later and disappeared only slowly, illustrating the lower initial rate of uptake of both the first and second bead. We therefore conclude that both Gα4-null and Gβ-null strains are inefficient in an early step of phagocytosis, unambiguously implicating a Gα in this mechanism. A similar functional analysis can now be performed for the proteins of unknown function in these groups with the aim of both validating our approach and strengthening the understanding of the role of heterotrimeric G proteins in phagocytic uptake. The strategy can equally be expanded to any other group of phagosome proteins.

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To whom correspondence should be addressed. Tel.: 41-22-379-6496; Fax: 41-22-379-6470; E-mail: thierry.solodati@biochem.unige.ch.

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