Selective membrane exclusion in phagocytic and macropinocytic cups

Valentina Mercanti1,*, Steve J. Charette1,*, Nelly Bennett2, Jean-Jeacques Ryckewaert3, François Letourneur4 and Pierre Cosson1,‡

1Université de Genève, Centre Médical Universitaire, Département de Physiologie Cellulaire et Métabolisme, 1 rue Michel Servet, CH-1211 Genève 4, Switzerland
2Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Département de Réponse et Dynamique Cellulaires, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France
3Laboratoire de Chimie des Protéines, ERM 201 INSERM/CEA/UJF, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France
4Institut de Biologie et Chimie des Protéines (IBCP UMR 5086), CNRS Université de Lyon, IFR128 BioSciences Lyon-Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, France

*These authors contributed equally to this work
‡Author for correspondence (e-mail: pierre.cosson@medecine.unige.ch)

Summary

Specialized eukaryotic cells can ingest large particles and sequester them within membrane-delimited phagosomes. Many studies have described the delivery of lysosomal proteins to the phagosome, but little is known about membrane sorting during the early stages of phagosome formation. Here we used Dictyostelium discoideum amoebae to analyze the membrane composition of newly formed phagosomes. The membrane delimiting the closing phagocytic cup was essentially derived from the plasma membrane, but a subgroup of proteins was specifically excluded. Interestingly the same phenomenon was observed during the formation of macropinosomes, suggesting that the same sorting mechanisms are at play during phagocytosis and macropinocytosis. Analysis of mutant strains revealed that clathrin-associated adaptor complexes AP-1, -2 and -3 were not necessary for this selective exclusion and, accordingly, ultrastructural analysis revealed no evidence for vesicular transport around phagocytic cups. Our results suggest the existence of a new, as yet uncharacterized, sorting mechanism in phagocytic and macropinocytic cups.

Key words: Membrane sorting, Phagocytosis, Macropinocytosis, Clathrin, Dictyostelium discoideum

Introduction

Phagocytosis is the process by which specialized eukaryotic cells internalize large particles (typically >500 nm in diameter). Cell surface receptors bind particles, initiate local intracellular signals and reorganize the actin cytoskeleton to induce the change in cell shape needed to engulf the particle (Mellman, 1996). A large portion of the plasma membrane is internalized during the phagocytosis of a particle, and in the absence of membrane sorting this would lead to an alteration of the specific composition of intracellular compartments. To avoid the indiscriminate presence of surface markers in endosomal/phagosomal compartments of phagocytic cells, efficient sorting should accompany the formation of phagosomes or selective membrane recycling of the engulfed cell surface markers should follow soon thereafter. A similar issue can be raised for cells engaged in macropinocytosis, where large endocytic vacuoles (typically >500 nm in diameter) form continuously, resulting in the engulfment of large amounts of both extracellular medium and plasma membrane. At the mechanistic level, phagocytosis and macroinocytosis present many similarities including the involvement of phosphoinositol phosphate signaling and actin cytoskeleton reorganization (Dormann et al., 2004; Lee and Knecht, 2002; May and Machesky, 2001). It is not clear however if the problem of membrane sorting is dealt with in a similar manner in both cases. In mammals, a few specialized cell types are actively engaged in either phagocytosis (macrophages, neutrophils, dendritic cells) or macroinocytosis (dendritic cells). Although they play key roles in various facets of the immune response, membrane sorting in these cells has been less extensively studied than in non-phagocytic cell lines like fibroblasts.

Many studies on membrane sorting in phagocytic cells have focused on the acquisition of endosomal/lysosomal markers in early phagosomes, a process that is initiated a few minutes after phagosome closure (Scott et al., 2003; Vieira et al., 2002). Comparatively little attention has been paid to the fate of plasma membrane proteins during or immediately after the formation of phagosomes, and it is often assumed that the composition of newly formed phagosomes essentially reflects the composition of the plasma membrane. A few studies however have suggested that some surface molecules might be specifically excluded from phagocytic cups. Specifically, major histocompatibility complex molecules (Clemens and Horwitz, 1992), VLA4 integrin, and Thy1 (Pierini et al., 1996) have been identified as molecules excluded from phagocytic cups, while concanavalin receptors might be concentrated (Berlin and Oliver, 1978). In addition, phagocytic cups might receive membrane from internal compartments, in particular endosomes (Bajno et al., 2000; Braun et al., 2004) and the endoplasmic reticulum (Gagnon et al., 2002), although the quantitative importance of these contributions remains to be
determined and might vary in different cell types (Touret et al., 2005). To our knowledge no data is available concerning the membrane composition of macropinosomic cups.

*Dictyostelium discoideum*, a soil amoeba, is a very active phagocytic and macropinocytic organism. It is a widely used model for the study of both phagocytosis and macropinocytosis (Duhon and Cardelli, 2002; Maniak, 2003) and allows comparative analysis of these two processes. Here, we followed the fate of several plasma membrane proteins in *Dictyostelium discoideum* and observed that some of them were excluded from phagocytic and macropinocytic cups. Our results suggest that selective exclusion was caused by specific restrictions to lateral diffusion in nascent phagosomes and macropinosomes, rather than by selective retrieval by vesicular transport.

**Results**

**Newly formed phagosomes are essentially derived from the plasma membrane**

The first aim of this study was to follow the fate of a collection of membrane markers at very early stages in the phagocytic pathway. For this, it was important unambiguously to identify phagocytic cups and newly formed phagosomes, and to distinguish them from more mature phagosomes. To this end, we used *Dictyostelium* cells expressing the PH domain of a cytosolic regulator of adenylcyclase (CRAC), fused to a green fluorescent protein (GFP) reporter. CRAC-GFP binds to phosphatidylinositol 3,4-bisphosphate and to phosphatidylinositol 3,4,5-trisphosphate and is recruited to the plasma membrane in regions involved in the formation of pseudopods, phagosomes and macropinosomes (Parent et al., 1998; Tuxworth et al., 2001). To determine precisely the kinetics of phagosome formation, cells were incubated with fluorescent yeast particles for defined periods of time. CRAC-GFP-positive phagocytic cups were detectable 1 minute after initiation of phagocytosis; closed CRAC-GFP-positive phagosomes after 2 minutes; and CRAC-GFP-negative phagosomes after 3 minutes (Fig. 1A,B). Thus the presence of CRAC-GFP is a hallmark of phagocytic cups and newly formed phagosomes (<3 minutes). These results are in good agreement with previous reports (Dormann et al., 2004; Parent et al., 1998), although different strains and experimental conditions were used by these investigators. In this study, CRAC-GFP negative phagosomes correspond to phagosomes 3-10 minutes after their initiation, since the cells were fixed after at most 10 minutes of phagocytosis. In agreement with the literature, these phagosomes are called maturing early phagosomes (Scott et al., 2003).

Several studies have indicated that membrane from internal compartments might be delivered to phagocytic cups (Bajno et al., 2000; Gagnon et al., 2002; Braun et al., 2004), potentially contributing to their growth. H72 protein, as shown in Fig. 3A,B, is present in phagocytic cups and newly formed phagosomes (Fig. 2A and data not shown). The accumulation of H72 protein in phagosomes before the loss of CRAC-GFP, suggesting that the first markers delivered to maturing phagosomes (Clarke et al., 2002). As observed by Clarke et al., we did not detect it in either phagocytic cups or in newly formed phagosomes (Fig. 2A). These results suggest that in the experimental conditions used in this study, there is no massive delivery of endosomal or endoplasmic reticulum membranes to the phagocytic cup and to the newly formed phagosome. This is in agreement with recent results that detected no delivery of ER membrane to phagocytic cups or phagosomes (Lu and Clarke, 2005; Touret et al., 2005). Massive delivery of internal membranes of endosomal origin was not initiated in newly formed phagosomes before the loss of CRAC-GFP, suggesting that both phagocytic cups and newly formed phagosomes are mostly derived from the plasma membrane. Consequently, we analyzed the membrane composition of phagocytic cups, of newly formed phagosomes, and of maturing early phagosomes separately.

**Differential membrane sorting in phagocytic cups**

Monoclonal antibodies specific for *Dictyostelium* membrane proteins (Ravanel et al., 2001) were used to monitor the abundance of various plasma membrane proteins in phagocytic structures. H72 detects a membrane-associated protein of 25 kDa localized mostly at the cell surface (Fig. 3A) (Ravanel et al., 2001). The identity of this protein is as yet unknown. The H72 protein, as shown in Fig. 3A,B, is present in phagocytic

![Fig. 1. Phagosome formation in cells expressing the CRAC-GFP protein.](Image)
cups and in newly formed phagosomes (CRAC-GFP positive) at a concentration similar to that detected at the plasma membrane. Its concentration decreased markedly only in maturing early phagosomes (CRAC-GFP negative).

The H161 antibody recognizes an 80 kDa protein which exhibits three transmembrane domains and shares homology with the human CTR1 zinc transporter (Ravanel et al., 2001). Contrary to the H72 protein, the H161 protein is mainly restricted to late endosomes even though a significant amount is found at the plasma membrane (Ravanel et al., 2001). Like the H72 protein, the H161 protein was present in phagocytic cups and in newly formed phagosomes at the same concentration (Fig. 3C). However, contrary to H72, the concentration of H161 increased markedly in maturing early phagosomes. The wide range of H161 concentrations in maturing phagosomes presumably correspond to various stages of maturation between 3 and 10 minutes after phagosome closure. These observations suggest that differential sorting of
H72 and H161 proteins in the phagocytic pathway of *Dictyostelium* is only initiated in maturing early phagosomes.

To determine whether the H72 and H161 proteins found in newly formed phagosomes originated from the cell surface and not from intracellular compartments, the cell surface was labeled with the corresponding antibodies and then the cells were allowed to engulf yeast particles for 3 minutes before fixing and labeling them with a fluorescent secondary antibody (see Fig. 3D). In this experimental setup, the H72 and H161 proteins were detected at similar levels in newly formed phagosomes and at the cell surface (Fig. 3E). This indicates that the H72 and H161 proteins observed in phagocytic cups and in newly formed phagosomes are indeed surface proteins captured during the formation of phagosomes and do not originate from intracellular compartments during phagosome formation.

The sorting of two other membrane proteins in the phagocytic pathway was analyzed in a similar manner and revealed a different profile. These two proteins are recognized by monoclonal antibodies PM4C4 and H36, respectively. They are both mostly present at the surface of *Dictyostelium* cells (Fig. 4A). Although their identity is unknown, biochemical analysis indicated that they are distinct proteins with a molecular weight of approximately 70 kDa and 46 kDa, respectively (Fig. 4B).

Both PM4C4 and H36 proteins were excluded from the membrane of phagocytic cups and of phagosomes (Fig. 4C-E). This exclusion was seen in all the phagocytic cups, but was more evident in mature cups nearing closure and characterized by a more invaginated profile than in shallow phagocytic cups. Note that the term exclusion does not prejudge whether markers were absent from phagocytic cups due to a selective entry block, or to an active ejection from the cup. The putative mechanisms responsible for selective exclusion are discussed in the Discussion section. Quantitative analysis revealed that exclusion of these two membrane proteins was completed essentially before the closure of phagocytic cups, since the concentration of PM4C4 and H36 in mature phagocytic cups was similar to that seen in closed phagosomes. These observations suggest that although they are mostly derived from the plasma membrane, phagocytic cups are a site of efficient membrane sorting.

**Similar sorting patterns in phagosomes and macropinosomes**

Macropinocytosis is a process similar to phagocytosis, both morphologically and biochemically (Dormann et al., 2004), but membrane sorting in macropinosomes has been poorly studied to date. In *Dictyostelium*, macropinocytic cups and macropinosomes form constantly, and they can be revealed by the recruitment of CRAC-GFP. Live analysis of macropinocytosis revealed that the CRAC-GFP-positive vacuoles in *Dictyostelium* cells correspond to newly formed macropinosomes (<1 minute) (Blanc et al., 2005). Since newly formed macropinosomes can be identified in this manner, their membrane composition can be analyzed by immunofluorescence. As shown in Fig. 5, the concentration of H72 and H161 proteins in newly formed macropinosomes was very similar to their concentration at the cell surface. On the contrary, PM4C4 and H36 proteins were largely depleted from newly formed macropinosomes (Fig. 5A,B).

To determine the origin of the H72 and H161 proteins present in newly formed macropinosomes, the surface of cells was prelabeled with the corresponding antibodies at 4°C and then the cells were incubated for 3 minutes at room temperature.
temperature before fixation. Immunofluorescence analysis revealed that the H72 and H161 proteins present in newly formed macropinosomes originated from the plasma membrane and not from intracellular compartments (Fig. 5C).

In addition H⁺-ATPase was also not detected in newly formed macropinosomes (Fig. 5D), in agreement with previous studies indicating that acidification of macropinosomes is initiated approximately 1 minute after closure of macropinosomes, when CRAC-GFP is no longer associated with the macropinosome (Blanc et al., 2005; Maniak, 2003). Together, these results indicate that the pattern of membrane proteins found in newly formed macropinosomes was identical to that seen in newly formed phagosomes. This suggests that sorting in the early compartments of the macropinocytic pathway is similar to sorting in phagocytic compartments.

To verify that the absence of some markers in the macropinosomes and the phagosomes was not due to rapid and selective degradation, we measured the stability of the H36 protein. For this, the cell surface was labeled with biotin at 4°C and the cells warmed for various periods of time. Lysates from these cells were immunoprecipitated with the H36 antibody and the amount of biotinylated H36 protein determined. No significant degradation of cell surface H36 was seen over a period of 1 hour (Fig. 5E). Since macropinosomes formed during that period of time represent a surface equivalent to the total cell surface (Hacker et al., 1997), the absence of H36 in newly formed macropinosomes could not be accounted for by a selective degradation.

Selective exclusion from macropinocytic cups

Macropinocytic cups are difficult to identify unambiguously because they are not characterized by the presence of phagocytic particles and it can be difficult to distinguish large folds of the plasma membrane from macropinocytic cups. Even the presence of CRAC-GFP at the level of the membrane is not sufficient per se to identify a macropinocytic cup, because pseudopods are also CRAC-GFP-positive. However, live imaging of cells expressing CRAC-GFP revealed that a particular morphology can be observed during the formation of a macropinocytic cup. Indeed 65% (n=64) of the macropinocytic cups observed adopt a characteristic U shape (for a detailed description see Fig. 6A) in the last 15 seconds (±6 seconds) preceding closure. The remaining 35% of the macropinocytic cups displayed various morphologies that could not be clearly distinguished from other structures (e.g. pseudopods) and were not taken into account in our analysis. Conversely, observation of live cells revealed that 82% of U-shaped CRAC-GFP positive structures correspond to macropinocytic cups within 20 seconds of their closure, indicating that macropinocytic cups can be identified reliably even in fixed cells following these criteria. This characterization opened the possibility of analyzing the membrane composition of macropinocytic cups by immunofluorescence in fixed cells.

The concentration of H36 and of PM4C4 in macropinocytic cups was markedly decreased compared with concentration at the cell surface, while the concentration of H72 and H161 proteins remained equivalent to the concentration observed at the plasma membrane (Fig. 6B). A more detailed analysis of H36 staining was conducted on individual macropinocytic cups. The decrease of H36 staining was more pronounced at the bottom of the cups, where the H36 labeling showed an intensity comparable to that seen in closed, newly formed, macropinosomes (Fig. 6C). Similar results were obtained with PM4C4 (data not shown). This suggests that the exclusion of

Fig. 5. Membrane sorting in macropinosomes. (A,B,D) Cells expressing CRAC-GFP were fixed and processed for total immunofluorescence using different antibodies. (A) Representative pictures of newly formed macropinosomes (green) stained with H36, PM4C4, H72 or H161 antibodies (white). (B) Relative intensity of H36, PM4C4, H72 and H161 staining in newly formed macropinosomes was determined and plotted as described in the legend to Fig. 3. H36 and PM4C4 staining was strongly reduced in newly formed macropinosomes. H72 and H161 staining was not. (C) H72 and H161 proteins found in the macropinosomes originate from the plasma membrane. Cells were incubated with H72 or H161 antibodies for 5 minutes at 4°C, washed and allowed to perform macropinocytosis for 3 minutes. Cells were then fixed, permeabilized and processed for immunofluorescence. (D) H⁺-ATPase (white) was not detected in newly formed macropinosomes (green). (E) The cell surface was biotinylated and cells incubated for 0 or 1 hour. Cells were then lysed, the H36 protein was immunoprecipitated, migrated on a polyacrylamide gel, transferred to nitrocellulose and revealed with the H36 antibody (total H36), or with avidin (biot. H36). A control where no antibody was used for the immunoprecipitation (w/o Ab) confirmed the specificity of the immunoprecipitation. Biotinylated surface H36 was not degraded during macropinocytosis. Bars, 4 μm.
H36 might actually be completed by the time the macropinosome is closing. Interestingly, the concentration of H36 was not diminished in areas of the plasma membrane that were not involved in the formation of a macropinosome, but where CRAC-GFP accumulated, such as pseudopods (Fig. 6C). These results suggest that macropinocytic cups represent distinct entities, morphologically and biochemically distinct from other regions of the plasma membrane engaged in signaling and active remodeling.

Adaptor proteins and myosins are not involved in selective exclusion

Proteins depleted from phagocytic or macropinocytic cups could be either retrieved selectively by vesicular transport, or sorted by lateral diffusion in the plane of the membrane. To evaluate the putative involvement of vesicles, we analyzed membrane sorting in knockout cells lacking the μ chain of the adaptor protein complexes 1, 2 or 3 (μ1, μ2 and μ3). These complexes are involved in the selection of cargo during the formation of clathrin-coated vesicles (for a review, see Owen et al., 2004). Exclusion of H36 or PM4C4 from macropinosomes (Fig. 7) and phagosomes (data not shown) was normal in these three mutant cell lines, suggesting that clathrin-coated vesicles do not participate in selective exclusion of H36 and PM4C4 from the cups. In agreement with this, we failed to detect any budding profile at the ultrastructural level that could indicate formation of vesicles in phagocytic cups (Fig. 8) after analysis of 21 different phagocytic cups representing a total of 188.8 μm of phagocytic membrane. Similarly, we observed no evidence of fusion of incoming vesicles. Ribosome-studded endoplasmic reticulum cisternae were occasionally detected in the vicinity of phagocytic cups, but we never observed fusion with the phagocytic cup membranes (Fig. 8). In fact, it is difficult to envisage how vesicles or the endoplasmic reticulum could traverse the dense and continuous layer of actin filaments surrounding the phagocytic cup.

We also tested the role of class I myosins B (myoB) and K (myoK) as well as class VII myosin (myoVII), which have been proposed to play a direct or indirect role in phagocytosis and macropinocytosis (Novak et al., 1995; Schwarz et al., 2000; Tuxworth et al., 2001). More specifically, myosin IB has been implicated in membrane sorting in the macropinocytic pathway (Neuhaus and Soldati, 2000) based notably on the observation that PM4C4 accumulated in endocytic vesicles in myoB knockout cells (Neuhaus and Soldati, 2000). We did not detect any change in the composition of newly formed macropinosomes in myoB mutant cells (Fig. 7), suggesting that this protein does not play a role in membrane sorting during formation of macropinosomes. Surprisingly, in contradiction with previous observations (Neuhaus and Soldati, 2000), we did not observe either any change in the intracellular localization of PM4C4 in these mutant cells. This may be due to the fact that our myoB mutants were obtained in a strain different from that used in previous studies (Neuhaus and...
Myosin IK and myosin VII have been implicated in phagocytosis and accumulate around the phagocytic cup (Schwarz et al., 2000; Tuxworth et al., 2001). The corresponding knockout cells were analyzed, and no anomaly in sorting of H36 or PM4C4 was detected in newly formed macropinosomes (Fig. 7) or phagosomes (data not shown). In summary, none of the myosin mutants tested (IB, IK and VII) appeared to be necessary for membrane sorting during the formation of macropinosomes.

**Discussion**

In this work we followed the fate of a panel of membrane proteins during phagocytosis and macropinocytosis. In cells expressing a CRAC-GFP fusion protein, we were able to identify unambiguously phagocytic cups, as well as newly formed phagosomes. We did not observe a massive delivery of internal membranes of endosomal or ER origin at very early stages of phagosome formation. Furthermore, several markers originating from the cell surface were present in newly formed phagosomes at concentrations similar to that seen at the cell surface. This observation also suggests that in phagocytic cups and newly formed phagosomes, there was no significant dilution of plasma membrane markers by internal membranes. Finally ultrastructural analysis did not reveal fusion of incoming vesicles at the level of the phagocytic cup. Our observations do not exclude the possibility that a small amount of membrane from intracellular compartments might be delivered to phagocytic cups but suggest that no massive delivery occurred. The maturation process characterized by the delivery of endosomal proteins and the removal of some endocytosed markers was initiated at a later stage, several minutes after closure of the phagosome.

Although phagocytic cups and newly formed phagosomes appeared essentially derived from the cell surface, their composition did not simply reflect that of the plasma membrane. Specifically, two membrane markers analyzed here (H36 and PM4C4) were excluded from phagocytic cups and were found only at very low levels in newly formed phagosomes. This sorting was highly specific, since two other markers (H72 and H161) were present at the cell surface and in newly formed phagosomes at similar concentration. Thus phagocytic cups are a site of rapid and specific membrane exclusion, defining a membrane domain with a composition different from that of the plasma membrane from which they are derived.

Since *Dictyostelium* amoebae perform both efficient phagocytosis and macropinocytosis we compared membrane sorting accompanying these two events. We also observed selective exclusion of membrane proteins in macropinocytic cups, and in newly formed macropinosomes, exhibiting the same pattern as observed in the phagocytic pathway. These results strongly suggest that the same molecular mechanisms control selective membrane exclusion during phagosome and macropinosome formation. Moreover, our results indicate that macropinocytic cups are a distinct entity of the cell surface, as selective exclusion was not observed in other regions of the plasma membrane engaged in active remodeling of the actin cytoskeleton, such as pseudopods.

We have obtained a few indications concerning the molecular mechanisms allowing selective membrane exclusion in phagocytic and macropinocytic cups. Ultrastructural analysis did not reveal vesicles budding at the level of phagocytic cups. Mutants affecting clathrin-associated adaptor proteins failed to show any defect in selective membrane exclusion in the cups. More specifically, we have shown previously that AP1 is associated with the phagocytic cup, and

**Fig. 7.** Composition of newly formed macropinosomes in mutant cells. Mutant cells (µ1, µ2, µ3, myoVII, myoK and myoB) expressing CRAC-GFP were fixed and processed for immunofluorescence using H36 (A) or PM4C4 (B) antibodies. Graphs present the relative intensity of H36 and PM4C4 in newly formed macropinosomes, determined and plotted as described in legend to Fig. 3.

**Fig. 8.** Absence of massive vesicular transport around phagocytic cups. Cells were allowed to engulf yeast particles for ten minutes, then fixed and processed for electron microscopy. The left panel is a representative image of a cell (C) with a phagocytic cup containing a yeast particle (Y). The right panel is a magnification of the box in the left panel. The phagocytic cup is surrounded by a continuous layer of F-actin (arrows) and presents no evidence for fusion or fission of vesicles. An endoplasmic reticulum cistern (arrowheads) is visible in the vicinity of the phagocytic cup but no direct contact was observed. Bar, 0.5 µm.
that cells expressing no \(\mu\)E exhibit a noticeable defect in phagocytosis (Lefkir et al., 2004). Many other defects were observed in the organization and function of the endocytic and phagocytic pathway in these mutant cells (Lefkir et al., 2004) (data not shown). It is also clear from the literature that AP1 contributes to membrane sorting in clathrin-coated vesicles (Owen et al., 2004), yet no defect was observed in our studies of the composition of newly formed macropinosomes in \(\mu\)L knockout cells. This suggests that AP1 does not contribute to the selective exclusion process. Similarly \(\mu\alpha\) and \(\mu\beta\) knockout cells exhibited unaltered sorting in the newly formed macropinosomes. Our results suggest that a mechanism other than vesicular transport is responsible for selective exclusion from phagocytic and macropinocytic cups. We propose that phagocytic and macropinocytic cups form a specific subdomain of the plasma membrane and that a subset of proteins is excluded from these structures by lateral diffusion in the plane of the membrane. Since the actin cytoskeleton is abundant around phagocytic and macropinocytic cups, proteins associated with the actin cytoskeleton may contribute to the formation of this specialized membrane domain. We speculate that lateral interactions in the plane of the membrane might account for selective exclusion in phagocytic and macropinocytic cups. Membrane anchors (transmembrane domains or lipid anchors) might be recognized by a sorting machinery in the plane of the membrane, restricting their access to certain specialized membrane domains. This compelled us to test the role of three myosin molecules implicated in phagocytosis. Myosin IK and myosin VII are present around phagocytic cups, while myosin IB was previously identified in the recycling of membrane from endosomal structures to the cell surface (Neuhauß and Soldati, 2000). We did not detect any alteration of the composition of newly formed phagosomes in the corresponding knockout mutant strains, suggesting that these proteins do not play a role in membrane sorting in the early macropinocytic pathway.

Altogether our results indicate the existence of a new, as yet undescribed mechanism of selective membrane exclusion, specific to the phagocytic and macropinocytic cup. It is difficult to compare our observations to the well-studied mechanisms of membrane sorting accompanying formation of transport vesicles. Indeed, very little is known about exclusion of membrane proteins from transport vesicles since recent work in that field focused rather on the selective concentration of membrane proteins caused by interactions with components of the cytosolic coat. Further analysis will be needed to identify molecular mechanisms ensuring selective membrane exclusion in newly formed phagosomes and macropinosomes. The fact that \textit{Dictyostelium} is suitable for genetic analysis should allow us to extensively test the role of various gene products in this new sorting process.

**Materials and Methods**

**Cells and reagents**

\textit{Dictyostelium discoideum} cells were grown at 21°C in HL5 medium (14.3 g l\(^{-1}\) peptone (Oxoid LTD, Basingstoke, Hampshire, UK), 7.1 g l\(^{-1}\) yeast extract (Gibco BRL, Dénf, Basel, Switzerland), 18 g l\(^{-1}\) maltose (Fluka, Buchs, Switzerland), 3.6 mM NaH\(_2\)PO\(_4\), and 3.6 mM KH\(_2\)PO\(_4\), pH 6.7). All \textit{Dictyostelium} cells used in this study were derived from the subclone DH1-10 (Cornillon et al., 2000) of the DH1 strain (Caterina et al., 1994). The \(\mu\) (Lefkir et al., 2003) and \(\mu\)VII (Gebbie et al., 2004) knockout strains have been described previously. The \(\mu\)K (Schwarz et al., 2000) knockout cells were a gift from T. Soldati (University of Geneva, Switzerland). The \(\mu\)B knockout strain (Novak et al., 1995) was obtained for this study by transfection of DH1-10 with the pDTb35R plasmid, a gift of M. Titus (University of Minnesota, Minneapolis, USA). The \(\mu\)B mutant clones were confirmed by PCR analysis of genomic DNA (Gebbie et al., 2004). To obtain the \(\mu\)2 and \(\mu\)3 knockout vectors, DNA fragments were amplified by PCR from genomic DNA and cloned into a pBluescript vector (Stratagene, La Jolla, CA). The amplified DNA fragments comprised nucleotides 1-661 and 778-1665 of the \(\mu\)2 coding sequence and nucleotides 200-388 and 1115-1505 of the \(\mu\)3 coding sequence. The \(\mu\)2 and \(\mu\)3 knockout constructs were built by inserting a blasticidin resistance cassette between the two cloned fragments. The final plasmids were linearized by digestion with restriction enzymes (SalI and NotI) and electroporated into DH1-10 cells (Alibaud et al., 2003). Transformants were selected in the presence of 10 \(\mu\)g ml\(^{-1}\) blasticidin and individual colonies were tested by PCR. Absence of expression of the \(\mu\)2 mRNA was then verified by RTPCR in \(\mu\)2 knockout clones. The \(\mu\)3 knockout were verified by Southern blot analysis of individual clones.

Cells constitutively expressing the PH domain of cytosolic regulator of adenylylcyclase fused to green fluorescent protein (CRAC-GFP) were generated by transfection with the WFS8 plasmid (Parent et al., 1998), a gift from P. Devreotes (Johns Hopkins Medical Institutions, Baltimore, USA). Transfected cells were grown in the presence of 10 \(\mu\)g ml\(^{-1}\) of G418 and transferred to G418-free medium three days before usage.

Mouse monoclonal antibodies against the p80 endosomal marker (H161), the p25 protein (H72), the subunit of the vacular H\(^{+}\)-ATPase (221-35-2) and a plasma membrane marker (PM4C4) were described previously (Neuhauß and Soldati, 2000; Ravanel et al., 2001). Protein Disulfide Isomerase was detected using 221-64-1, a mouse monoclonal antibody (Monnat et al., 1997). G. Gerisch (Max-Planck-Institut, Martinsried, Germany) provided 221-35-2 and 221-64-1 antibodies while PM4C4 antibody was a gift from J. Garin (CEA Grenoble, France). The H36 mouse monoclonal antibody was obtained as described previously (Ravanel et al., 2001).

**Immunofluorescence**

Cells (0.5 × 10\(^{6}\)) were attached to a glass coverslip in fresh HL5 medium and incubated at 21°C for 3 hours, then fixed with 4% paraformaldehyde. After fixation, cells were permeabilized by incubation for two minutes in methanol at –20°C and then incubated with the indicated primary antibody in phosphate buffer containing 0.2% bovine serum albumin. The primary antibodies were analyzed using a secondary antibody against mouse IgG and coupled to Alexa Fluor 647 (Molecular Probes/Invitrogen, Eugene, OR). Cells were visualized with a LSM510 confocal microscope (Carl Zeiss, Feldbach, Switzerland). To study phagocytosis, rhodamine-labeled yeasts (Cornillon et al., 2000) were added to the medium 10 minutes before fixation. While paraformaldehyde fixation ensured optimal preservation of the structural integrity of the phagocytic cups, the methanol permeabilization was crucial, since permeabilization with saponin only partially permeabilized some of the intracellular compartments (data not shown). These observations account for the minor differences distinguishing our data from previous studies where cells were permeabilized with saponin (Ravanel et al., 2001).

We followed the fate of H161 proteins H72 and H161 localized at the cell surface, cells were incubated with the appropriate antibody for 5 minutes at 4°C, washed and incubated at 21°C for another 3 minutes before fixation, either in the presence or in the absence of yeast particles. Cells were then fixed and permeabilized as described above and then incubated with an anti-mouse antibody coupled to Alexa 488.

For quantitative analysis of images, the Metamorph 6.0 software was used (Universal Imaging Corporation, Downingtown, PA). The average fluorescence intensity of a hand drawn region delimiting the structures to be quantified was done. In the graphs each dot indicates the relative intensity of the average fluorescence in one macropinocytic or phagocytic structure compared with the average fluorescence of the plasma membrane in the same cell after substraction of the background (measured outside the cells). The values (in %) were rounded to the nearest multiple of 5 to simplify graphic presentation. The data set presented in the figures is the analysis of at least 20 cells. Each experiment was repeated and quantified at least 3 times with very similar results. Moreover key experiments were repeated using different immunofluorescence protocols to exclude the possibility of fixation or permeabilization-specific artifacts. When cells were fixed and permeabilized with methanol alone or with paraformaldehyde and saponin, we obtained results essentially identical to those presented here (data not shown).

**Protein electrophoresis and immunodetection**

Total protein extracts from \textit{Dictyostelium} cells were separated by electrophoresis (SDS-PAGE) in a 10% acrylamide gel under non-reducing conditions. Proteins were then transferred to a nitrocellulose membrane, which was incubated in a Tris-
buffered solution containing skin milk powder (7%). After blocking and appropriate washes, the membrane was incubated with the indicated monoclonal antibody, then with a horseradish-peroxidase-coupled anti-mouse secondary antibody (BioRad, Reinach, Switzerland). Bound antibody was visualized with ECL.

Cell surface biotinylation
Surface proteins of living cells were biotinylated using NHS-SulfoBiotin (Pierce Chemical, Rockford, IL) as previously described (Benghezal et al., 2003). To follow their fate, cells were incubated in HL5 at room temperature for 0 or 60 minutes and then lysed in phosphate buffer (pH 7.4) containing 1 mM EDTA, 1% NP40 and 1 mM Phenylmethylsulphonyl fluoride. Cell lysates were cleared by centrifugation, mixed with H36-coated Protein A-sepharose (Sigma, St Louis, MO) and incubated for 1 hour at 4°C. Beads were washed two times with the lysis buffer and mixed with 0.5 volume of SDS-PAGE loading buffer. An aliquot of each sample was separated on an acrylamide gel and immunodetected with H36 as described above. Another aliquot was separated on a gel and biotinylated proteins detected with ImmunoPure avidin coupled to horseradish peroxidase (Pierce Chemical, Rockford, IL) to detect biotinylated protein.

Electron microscopy
To analyze the morphology of phagocytic cups at the ultrastructural level, cells were incubated with yeast particles for 10 minutes, then fixed successively in HL5 medium containing 4% paraformaldehyde (30 minutes); in phosphate buffer (pH 7.4), containing 4% paraformaldehyde (30 minutes); in phosphate buffer containing 2% glutaraldehyde (30 minutes); in phosphate buffer containing 2% glutaraldehyde and 0.3% osmium tetroxide (30 minutes), and in 2% osmium tetroxide (4°C, 1 hour). Fixed cells were dehydrated and embedded in Epon resin and processed for conventional electron microscopy as described previously (Orci et al., 1973). Grids were examined in a Tecnai transmission electron microscope (FEI, Eindhoven, The Netherlands).

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