FEBS 17819

FEBS Letters 398 (1996) 37–42

Protein phosphorylation during phagosome maturation

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Received 14 September 1996; revised version received 15 October 1996

Abstract Phagolysosome biogenesis is driven by a series of interactions between phagosomes and organelles of the biosynthetic and endocytic pathways. The presence of endocytic markers on phagosomes suggests that phagosomes and endosomes share common structural and functional characteristics. In that line of thought, protein phosphorylation has been shown to be involved in regulatory aspects of the fusion properties of endosomes and other vacuolar organelles. To study further the mechanisms involved in phagolysosome biogenesis, we have investigated the presence of phagosome proteins that can be phosphorylated in vitro by endogenous phagosome-associated kinases. The results obtained show that proteins phosphorylated on tyrosine residues are present on phagosomes. Moreover, complex phosphorylation/dephosphorylation cycles appear to occur during phagolysosome biogenesis. The addition of endosome fractions to phagosomes inhibit the phosphorylation of phagosome proteins. These results suggest that phosphorylation and dephosphorylation events could play roles in the biogenesis of phagolysosomes and regulate, in part, the complex in vivo interactions between phagosomes and endosomes.

Key words: Phagocytosis; Lysosome; Phosphorylation; Macrophage

1. Introduction

Phagocytosis is the process used by a variety of cells to internalize large particulate materials such as bacteria or dust particles [1]. The internalization of these particles results in the formation of plasma membrane-derived organelles, the phagosomes. These compartments are transformed into phagolysosomes, where proteolytic degradation of the ingested materials occurs, by interacting with organelles of the endocytic and biosynthetic apparatus. Stahl and colleagues have shown that, within minutes of their formation, phagosomes are involved in fusion events with early endosomes [2–4]. These interactions are accompanied by rapid modifications of phagosome biochemical composition with the loss of recycling receptors and the acquisition of endocytic markers [5]. Subsequent studies have shown that phagosomes can also interact with late endosomes [6,7] and terminal lysosomes [8], enabling further transformation of phagosomes, over long periods of time.

Although the molecules and mechanisms involved in endosome functions are beginning to be understood, little is known about those of phagosomes. However, the fact that many of the proteins present on endosomes are also observed on highly purified phagosome preparations suggests that common mechanisms might be used by both organelles for their functions [7,9]. Among the proteins present on both organelles are LAMP1 and 2, annexins II and VI, the mannose 6-phosphate receptors, the β1 and β2 subunits of trimeric G proteins, as well as sets of as yet unidentified proteins observed using 2-D gel electrophoresis [7,10,8]. Endosomes and phagosomes also display GTPases of the ras-related family, among which rab5, rab7 and rap1 [7,11]. The first two were shown to be involved in regulatory processes during endosome fusion events, both in vivo and in vitro [12–14]. In contrast to endosomes, the ease with which latex-containing phagosomes can be purified allowed demonstration of the sequential association of these molecules to phagosomes at precise intervals during phagolysosome biogenesis suggesting their involvement in the fate of phagosome fusion events. However, the complexity of phagosome composition, highlighted by their two-dimensional electrophoretic protein profile displaying over 250 polypeptides [10,15], argues for the existence of multiple ways to regulate phagolysosome formation.

Recent data have shown that phosphorylation and dephosphorylation processes are involved in the regulation of various membrane traffic events, including endosome fusion [16–18] and the fusion of zymogen granules with the plasma membrane during exocytosis [19]. To investigate whether phosphorylation events could also be involved in phagolysosome biogenesis, we performed in vitro phosphorylation analyses to assess the presence of kinases and their substrates on phagosomes isolated at various time points after their formation. Our results show that different sets of proteins are phosphorylated and dephosphorylated at precise intervals during phagosome maturation, suggesting their involvement in a regulated way at key points during phagolysosome biogenesis. Moreover, the interactions of endosomes and phagosomes in vitro seem to influence the state of phagosome proteins phosphorylation. These data suggest that phosphorylation and dephosphorylation events may regulate some aspects of phagolysosome biogenesis.

2. Materials and methods

2.1. Phagosome formation and isolation

Phagosomes were formed in subconfluent cultures of J774 macrophages, by the internalization of latex beads 0.8 μm diluted 1:200 in culture medium as described previously [7]. Cells were then washed in cold phosphate-buffered saline. For kinetic studies, cells were then put back in culture for 0–48 h in medium without beads. For each time point, cells from two 10-cm Petri dishes were used at 80% confluency. Cells were then homogenized and the latex bead-containing phagosomes isolated on sucrose step gradients as described previously [7]. Endosomes were isolated from BHK-21 cells as described [20].

2.2. Western blot analysis

For Western blot analysis, phagosomes were isolated after 60 min of bead internalization followed or not by a 15 h chase in culture medium. Cell homogenization and phagosome purification were performed in the presence of phosphatase inhibitors (10 mM sodium

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PII S0014-5793(96)01213-6

fluoride, 10 mM tetrasodium pyrophosphate decahydrate, 10 mM β-glycerophosphate, 0.4 mM sodium vanadate, 2 mM EDTA in water, added to the homogenization buffer and all sucrose solutions) following the isolation procedure described previously [7]. Phagosome proteins were isolated by SDS-PAGE on 12% minigels and transferred to nitrocellulose following standard procedures. Anti-phosphotyrosine antibodies (monoclonal antibody PT66, Sigma, St. Louis, MO) were used following the manufacturer recommendations.

2.3. Phagosome in vitro phosphorylation

Before in vitro phosphorylation, purified phagosomes isolated on the sucrose gradients were resuspended in 10 μl of ice-cold phosphorylation buffer (250 mM sucrose, 3 mM imidazole (pH 7.4), 12.5 mM HEPES (pH 7.4), 1.0 mM 1,4-dithiothreitol (DTT), 50 mM KOAc (pH 7.0), 2 mM $MnCl_2$). For [γ-^{32}P]ATP labeling, each phagosome fraction was supplemented with 10 μCi [γ-^{32}P]ATP and incubated for 1 min at 37°C. The in vitro phosphorylation reactions were stopped by the addition of 50 μl boiling sample buffer for 1 min and the samples processed for SDS-PAGE using standard procedures. The number of phagosomes present in each preparation was determined in the flow cytometer by forward light angle scattering analysis and equal number loaded on gels at all time points [7]. The effect of the interactions between phagosomes and endosomes on the phosphorylation of their proteins was studied by gently mixing, prior to in vitro phosphorylation, an endosome fraction from BHK-21 cells with phagosomes formed by the internalization of latex beads for 60 min followed by a 60 min chase. The volume of each sample was adjusted to yield phosphorylated bands of similar intensity for each organelle after SDS-PAGE. Phosphorylation was then performed as above. After the reaction, 1 ml of ice-cold buffer without [γ-^{32}P]ATP was added, and phagosomes were isolated from the endosomes by centrifugation for 2 min at full speed in an Eppendorf centrifuge. For all experiments, the phagosome pellet was then boiled in sample buffer and processed for SDS-PAGE in 12.5% SDS gels. Gels may appear different in some experiments because of variations in the exposure time of the films.

3. Results

The fusion properties of endosomes appear to be regulated in part by phosphorylation and dephosphorylation of key proteins [16]. To investigate the possible involvement of this process in phagosome functions, we used an in vitro approach to show the presence of kinases on phagosomes and the nature of their substrates.

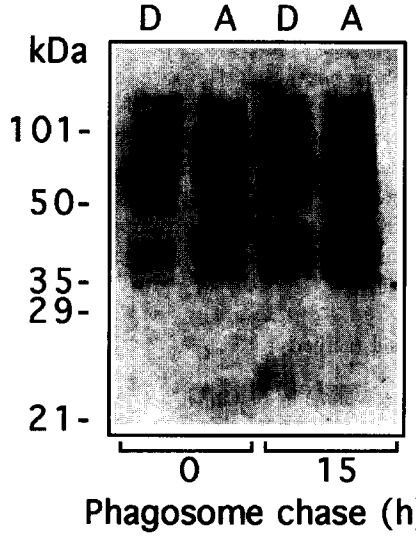

Fig. 1. Phosphotyrosine residues on phagosome proteins. Phagosomes were formed by the internalization of latex beads for 60 min followed by the indicated chase times. Phagosomes were then isolated on sucrose step gradients, and their protein separated according to their partition in Triton X-114. The proteins present in the aqueous (A) and detergent (D) phases were then separated by SDS-PAGE and transferred to nitrocellulose. Antiphosphotyrosine (monoclonal antibody # P66) was then used to reveal the presence of phosphotyrosine residues on phagosome proteins.

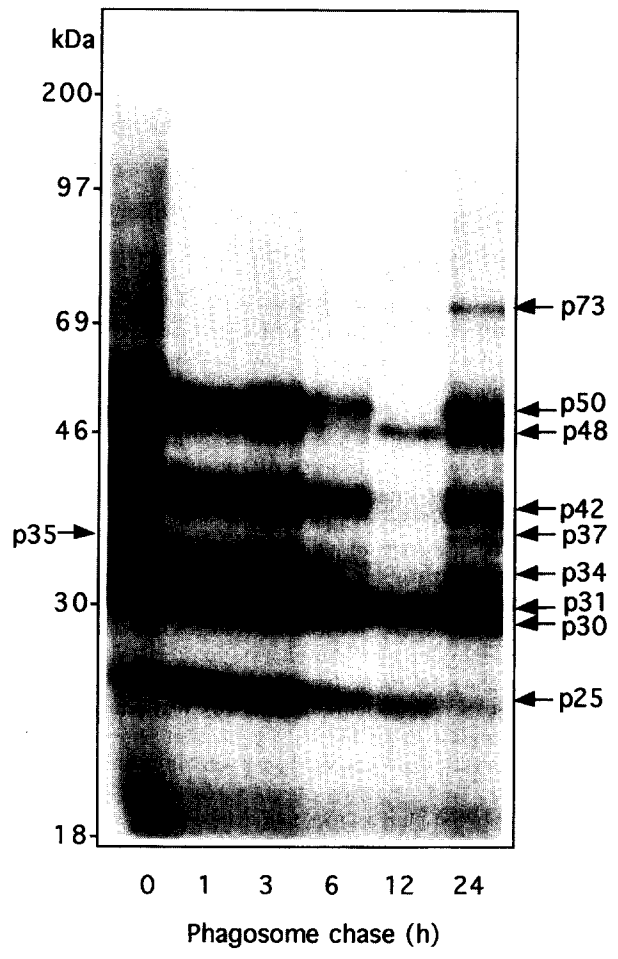

Fig. 2. Distribution of in vitro phosphorylated proteins of J774 macrophage phagosomes at various stages of phagolysosome biogenesis. Phagosomes were formed by the internalization of latex beads for 60 min followed by the indicated chase times. Phagosomes were then isolated on sucrose step gradients, and phosphorylation was performed in vitro for 60 sec at 37°C by the addition of 10 μM [g-^{32}P]ATP for each time point. No cytosol or exogenous kinases were added. The same number of phagosomes, determined by FACS, were analyzed at each time points. The samples were then processed for SDS-PAGE, the gels dried and exposed for 2 h on films. Ten distinct polypeptides were resolved, which are referred according to their relative migration positions. The molecular weight are shown in kDa.

First, we used an anti-phosphotyrosine antibody to assess the presence of such residues on phagosome proteins. This was done on phagosomes formed by the internalization of latex beads for 60 min followed or not by a 15 h chase to form early and late phagosomes [7,10]. The results obtained indicate the presence of several phagosome proteins phosphorylated on tyrosine residues. Most of these proteins are enriched compared to a crude cell homogenate (results not shown). To determine if these proteins are possibly associated to the phagosome membrane, we performed Triton X-114 extraction (Fig. 1) following the procedure of Bordier [21]. This allowed us to show that some of the phosphorylated

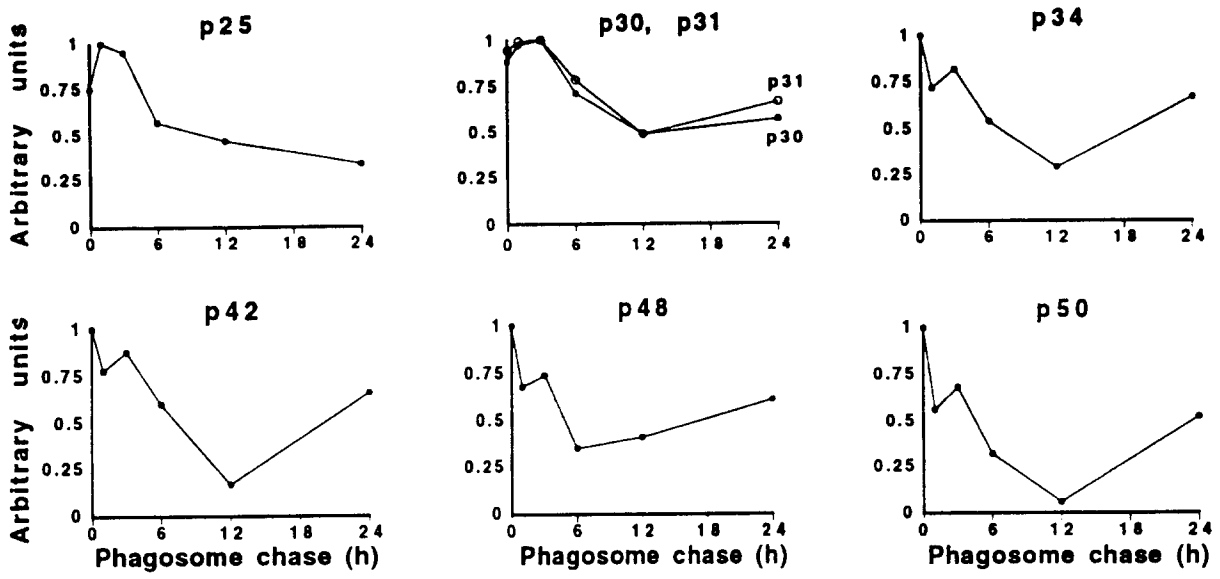

Fig. 3. Analysis of the relative intensity of phagosome-associated proteins, isolated at various steps of phagolysosome biogenesis, after in vitro phosphorylation. The results obtained in figure 2 were analyzed by densitometry and plotted in relative units with time. A value of 1.0 was assigned for each protein at the time point showing the highest density.

proteins display affinity for lipid bilayers, suggesting their potential involvement in phagosome fusion events.

Second, we performed in vitro phosphorylation studies on phagosomes isolated at various time points during phagolysosome biogenesis to show potential variation in phosphorylated proteins during this process. The results obtained indicate that phagosomes display different patterns of labeled polypeptides depending on their stage of maturation (Fig. 2). Since neither cytosol nor exogenous kinases were added to the purified phagosomes during the in vitro phosphorylation procedure, these proteins are probably phosphorylated by endogenous phagosome-associated kinases. Several bands between 25 and 73 kDa were observed on phagosomes isolated at each time point studied. These are referred to as p25, p30, p31, p34, p42, p48 and p50, according to their apparent migration after SDS-PAGE. In addition to these peptides, a band of 35 kDa (p35) was observed only on the earliest phagosome preparation studied, while two bands referred to as p37 and p73 were observed only on the late phagosomes (12–24 h). The latter peptides appear to be true late phagocytic markers since their intensity continued to increase on phagosomes isolated between 24 and 48 h after their formation (results not shown). The protein patterns presented in Fig. 2 were highly reproducible and could be obtained in four distinct experiments. The phosphorylation state of seven of the major phosphoproteins was followed with time by measuring the density of each band at each time point (Fig. 3). A striking observation was that the density of these proteins decreased on 6–12-h-old phagosomes and increased on 12–24-h-old phagosomes. The only exception to the latter trend was p25 for which the phosphorylation first increased for 1 h and decreased gradually as phagosomes became older.

The kinetic analysis of phagosome phosphorylation was also performed using manganese or magnesium as cation (Fig. 4). The results obtained show that the endogenous phagosome-associated kinase activity was optimal in the presence of manganese. In the presence of magnesium, only p50 was observed as a major phosphoprotein. Since tyrosine kinases have been shown to be more efficient when manganese is used as cation [22,23], our results confirm our Western blot analysis showing that some of the phagosome proteins are phosphorylated on tyrosine residues. In vitro phosphorylation was also performed in the presence of manganese or magnesium with [γ-^{32}P]GTP as a phosphate donor. The results indicate that under our conditions GTP is a poor phosphate donor since only a few faint bands could be observed (not shown), suggesting a specific ATP requirement by phagosome kinases. To evaluate the presence of endogenous phosphatase activity on phagosomes, we performed in vitro phosphorylation of an early phagosome preparation and further incubated the phosphorylated samples in medium containing a large excess of cold ATP for various time points at 37°C, to allow potential endogenous phagosome-associated phosphatases to act (Fig. 5). The results obtained showed that none of the bands phosphorylated after the initial reaction disappeared after up to 30 min of incubation with cold ATP. In contrast, when alkaline phosphatase was added to phosphorylated phagosomes, most of the phosphorylated peptides disappeared (Fig. 5). These results demonstrate the sensitivity of phagosome phosphoproteins to phosphatases and that little endogenous phosphatase activity is present in this 60 min phagosome preparation.

Interactions between phagosomes and endocytic organelles are believed to be required for the transformation of phagosomes into phagolysosomes [7]. To investigate whether these interactions could influence the phosphorylation of some of the phagosome proteins, we have isolated endosomes from BHK cells and mixed them with isolated phagosomes prior to the in vitro phosphorylation. After phosphorylation, the phagosomes were isolated from the bulk of the mixed endosomes and their phosphoprotein patterns analyzed (Fig. 6). We also analyzed the phosphoprotein patterns of endosomes and phagosomes alone. The results showed that the phosphoprotein pattern of BHK endosomes differs from that of phagosomes. When phosphorylation of phagosome proteins was performed in the presence of endosomes, most of the phosphoproteins normally observed on phagosomes were not phosphorylated. The polypeptide patterns were, rather, typical of endosomes, probably resulting from the residual binding of

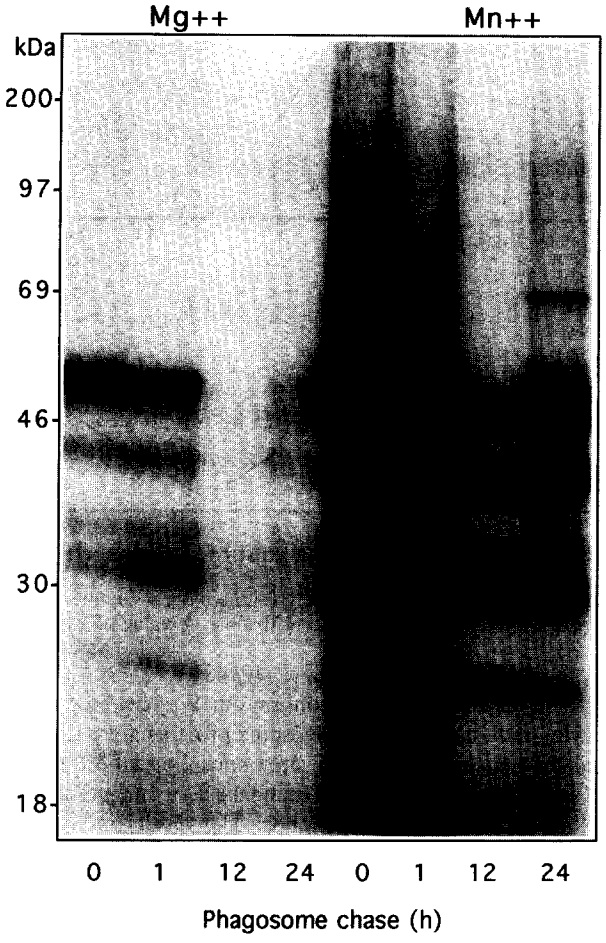

Fig. 4. Cation dependency of the in vitro phosphorylation process. In vitro phosphorylation was performed as described in figure 2 in buffer containing either magnesium or manganese as cations. The results show the low efficiency of the phosphorylation reaction in the presence of magnesium for each band except p50 which could be phosphorylated to a lower level.

endosomes to phagosomes. Whatever the precise mechanisms are, our data raise interesting questions showing that in vitro interactions of phagosomes with endosomes result in the inhibition of the phosphorylation of some of the phagosome proteins.

4. Discussion

We have proposed recently that the interactions between phagosomes and endocytic organelles could be regulated by the association of various members of the ras-related family of small GTP-binding proteins to these organelles [7,9]. Indeed, GTPases have been involved in the regulation of fusion events along the vacuolar apparatus (for reviews see [24–26]). However, we have just shown using Western blot analysis that a set of proteins involved in fusion events, including rabs, rabaptin-5 [27] and SNAREs [28] are associated to maturing phagosomes at all times, despite the finding that phagosome fusion properties are drastically modified during the same period. The molecules or mechanisms involved in the regulation of phagosome fusion properties are still unknown. The finding that phosphorylation events might regulate endosome fusion [16–18] prompted us to investigate the presence of kinases and phosphatases on phagosomes and the nature of their substrates. This is particularly interesting in light of the finding that some of the phagosome proteins, believed to play key roles in phagolysosome biogenesis, are phosphorylated in vivo at some stages. Among the notable examples are the rap1 protein [29], the annexin II protein [30] and the small mannose 6-phosphate receptor [31]. Moreover, phosphorylation of various proteins has been observed in response to the binding of particles to receptors at the cell surface during the early steps of phagocytosis (see [1]), a process believed to be important for the integration of signals between Fc receptors and the underlying cytoskeleton [32].

In the present study we have performed in vitro phosphorylation of proteins associated with J774 macrophage phagosomes isolated at various time points after their formation, to assess if phosphorylation and dephosphorylation processes occur during phagolysosome biogenesis. We report here that the transformation of a given organelle with time, the phagosome, is accompanied by the complex modulation of the content or the phosphorylation state of its phosphoproteins. The phagosome preparations isolated at different time points displayed distinct patterns of in vitro phosphorylated proteins, the most complex pattern showing nine major phosphopeptides. A recent study using a similar approach showed that distinct sets of proteins could be phosphorylated on various organelles [33], demonstrating the specificity of the technique. Although phagosomes were not analyzed in the latter study, the investigators showed that four major phosphoproteins

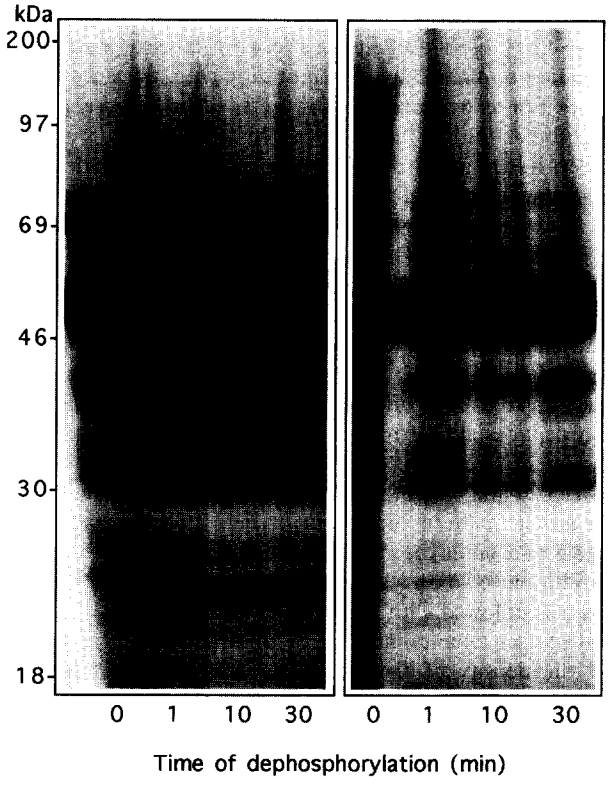

Fig. 5. Dephosphorylation of phagosome proteins by endogenous phosphatases or exogenously added alkaline phosphatase after in vitro phosphorylation. Phagosomes were processed for in vitro phosphorylation as in figure 2. Excess of cold ATP was then added to each sample together with (left panel) or without (right panel) 14 IU of alkaline phosphatase and the incubation continued for the indicated time points at 37°C. The results indicate the apparent absence of endogenous phosphatase activity in this phagosome preparation (60 min pulse and 60 min chase of beads).

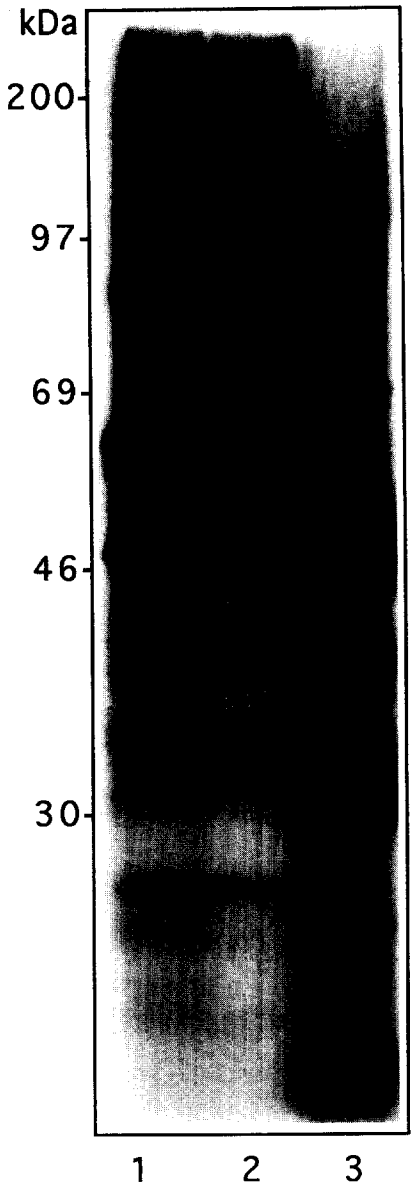

Fig. 6. Effect of the interaction of endosomes and phagosomes in vitro on the phosphorylation of their proteins. Endosomes were prepared as described in material and methods and mixed with phagosomes prior to the in vitro phosphorylation. Phosphorylation was then performed as in figure 2. After this step, 1 ml of incubation buffer containing an excess of cold ATP was added and phagosomes were separated by pelleting at 10 000 rpm in a table-top Eppendorf centrifuge for 2 min. The pellet of phagosomes was loaded in lane 2. Lane 1 contains early endosomes only. Lane 3 contains phagosomes not pre-incubated with endosomes. The results show that endosomes are able to inhibit the phosphorylation of most phagosome proteins when added to the in vitro assay.

were specifically associated with endosomes. In our phagosome preparations, we observed proteins of similar apparent molecular mass, together with other proteins that vary according to the phagosome age. The conditions used in the present study for in vitro phosphorylation indicate that phagosome proteins are phosphorylated by endogenous phagosome-associated kinases, that may include tyrosine kinases, as shown by Western blots with anti-phosphotyrosine antibodies (Fig. 1).

The patterns of appearance and disappearance of a set of ^{32}P-labeled proteins of similar molecular weight during phagolysosome biogenesis strongly suggest that these polypeptides are the same at the various time points studied, rather than unrelated proteins of similar molecular weight. Thus, it appears that phosphorylation and dephosphorylation of a relatively small set of polypeptides, at precise intervals during phagolysosome biogenesis, might play key roles in the regulation of phagosome maturation. Alternatively, the changes in the phospholabeling intensity of some of the phagosome proteins with time could be related to their association and dissociation from phagosomes, although the total protein profile of phagosomes do not seem to simplify with time [7].

The phosphorylation pattern changes observed in the present study indicate that latex beads are unlikely to reach rapidly a stable compartment after their internalization, as proposed earlier [6], but rather that phagosomes are continuously modified over long periods of time. A key process in the transformation of newly formed phagosomes into phagolysosomes appears to be their interactions with endocytic organelles [7]. These interactions allow the transfer to phagosomes of hydrolases enabling the digestion of the ingested particulate materials. Previous studies have shown that phagosomes can interact with terminal lysosomes [8], defined as lamp/lgp positive structures but devoid of recycling receptors and the rab7 protein [34,35], as well as with early endosomes [2,3] and with late endosomes, in a microtubule-dependent way [6,7]. Interestingly, the phosphorylation of CLIP-170, a protein that links endocytic vesicles to microtubules, inhibits the binding of these organelles [36,37]. The observation that mixing of endosomes and phagosomes in vitro resulted in the inhibition of phagosome protein phosphorylation provides further evidence that phosphorylation events might regulate the interactions between these organelles. The nature of this inhibition is unknown. It could be due to the presence of phosphatases on endosomes, since no obvious endogenous phosphatase activity could be detected on phagosomes under the conditions used in the present study. It could also be due to the interaction of phagosome proteins with components of endosomes resulting in the masking of the protein phosphorylation sites or to, as yet, other unknown mechanisms.

Together with the appearance and disappearance of various proteins on phagosomes with time, the continuous changes in the phosphorylation patterns of phagosome proteins strongly suggest that phosphorylation/dephosphorylation events may regulate key aspects of phagolysosome biogenesis. The nature and identity of the phagosome phosphorylated proteins are currently under investigation.

Acknowledgements: The authors wish to thank Jean Gruenberg and Gareth Griffiths for support. They also wish to thank Serge Roche and Sara Courtneidge for help and Rob Parton and Paul Clarke for critical reading of the manuscript. N.N.N. is the recipient of a studentship from the Fonds de la recherche en santé du Québec. M.D. is the recipient of a scholarship from the Fonds de la recherche en santé du Québec and a grant from the Medical Research Council of Canada.

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