Evidence for a pool of coronin in mammalian cells that is sensitive to PI 3-kinase

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Abstract Coronin, a 57 kDa actin binding protein elutes with an apparent molecular mass of 400–600 kDa from gel filtration columns. This fraction is not unrelated to the reported 200 kDa complex where coronin is associated with *phox* proteins of the NADPH-oxidase. Phosphatidylinositol 3-kinase (PI 3-kinase) solubilizes coronin from the 400–600 kDa complex, thus constitutive active PI 3-kinase is sufficient to disrupt the complex, whereas wortmannin stabilizes it. Conversely, the *phox* protein associated pool of coronin is PI 3-kinase independent. During phagocytosis coronin is recruited together with PI 3-kinase to membranes of nascent and early phagosomes co-localizing with the actin cytoskeleton, confirming that coronin contributes to phagocytosis. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Coronin; Phosphatidylinositol 3-kinase; Phagocytosis; Macrophage

1. Introduction

Coronin, a 57 kDa cytosolic protein, was originally described as an actin binding protein in *Dictyostelium discoideum* [1] and is expressed in hematopoietic cells [2–7]. The name was derived from its localization in crown-shaped extrusions formed by the growing amoebae [2]. It associates with the cortical actin cytoskeleton and is enriched at the leading edge where it contributes to the formation of nascent phagosomes [8]. Recently it was shown that in human neutrophils coronin co-elutes on gel filtration columns in a high molecular weight complex together with the cytosolic components, $p40^{phox}$, $p67^{phox}$ and $p47^{phox}$ of the NADPH oxidase [5]. Quantitative analysis revealed that coronin is very abundant in the cytosol of neutrophils where it binds specifically to $p40^{phox}$ [5]. Two possible functions were suggested: coronin acts as a linker between the cytosolic components of the oxidase and the cortical actin cytoskeleton and as a docking site for the translocation of *phox* proteins to the plasma membrane and of nascent phagosomes [5,9].

The respiratory burst of phagocytes is important for the efficient killing and elimination of microorganisms [10]. Upon stimulation the NADPH oxidase assembles at the plasma membrane and becomes internalized together with the phagocytic prey. Wortmannin, a selective phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor [11,12], attenuates superoxide formation, but its effect on phagocytosis is controversial [13–15]. In mammalian cells several PI 3-kinases have been characterized [16]. The α, β and δ isoforms, type I_A PI 3-kinases, are heterodimers consisting of a specific catalytic (p110) and a common regulatory (p85) subunit. Stimulation of cells by a number of growth factors, chemoattractants and phagocytic stimuli leads to activation of PI 3-kinase resulting in the rapid formation of 3-phosphoinositides ($PI(3,4,5)P_3$ and $PI(3,4)P_2$). PI 3-kinase activity has been implicated in the regulation of the actin cytoskeleton in receptor mediated signaling. The lipid kinase was shown to be a proximate effector of protein tyrosine kinases involved in growth factor mediated membrane ruffling [16]. Similarly, PI 3-kinase was implicated as downstream effector of tyrosine kinases in Fc receptor mediated phagocytosis [14,17,18]. Membrane targeting of PI 3-kinase by fusion of a HaRas-derived CAAX-box to the C-terminus of p110 results in stimulus independent $PI(3,4,5)P_3$ formation [19]. In p110CAAX transfected GM-128 cells, two-fold constitutively elevated $PI(3,4,5)P_3$ levels were observed which cause continuous activation of protein kinase B and the enhanced basal phosphorylation of the cytosolic component $p47^{phox}$ of the NADPH oxidase [19].

We provide evidence for a high molecular weight pool of coronin that is solubilized by PI 3-kinase activity and is independent of *phox* proteins. Immunofluorescence analysis suggests that PI 3-kinase and coronin co-operatively regulate early stages of phagosome formation.

2. Materials and methods

2.1. Materials

Cell culture media were purchased from Gibco-BRL. Horseradish peroxidase conjugated goat anti-rabbit IgG was obtained from Bio-Rad, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG from Santa Cruz Biotechnology. Rhodamin–phalloidin was from Sigma. Rabbit anti-p85 IgG was affinity purified on immobilized recombinant full-length human p85α [19]. Preparation of the anti-coronin rabbit serum was previously described [5]. Anti-$p47^{phox}$ antibody was kindly provided by Dr. O.T.G. Jones (University of Bristol, UK). 17-Hydroxywortmannin was a gift from T. Payne (Novartis Pharma, Basel, Switzerland).

2.2. Cell culture

J774A.1 murine macrophages, the human myeloid GM-1 and GM-128 expressing p110CAAX [19] were grown in RPMI-1640 medium supplemented with 10% fetal calf serum.

2.3. Cell fractionation and chromatography

GM-1 and GM-128 cells were serum-starved overnight, washed once in phosphate buffered saline (PBS) and resuspended (5×10^7

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cells/ml) in ice cold relaxation buffer (RB: 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM K^+PIPES, pH 7.3) containing 1 mM dithiothreitol (DTT), 2.5 mM diisopropyl fluorophosphate and a protease inhibitor cocktail (Complete, Boehringer). Cells were disrupted by N_2 cavitation at 300 psi for 20 min on ice. Nuclei and cell debris were removed from the homogenate by centrifugation ($1000\times g$ for 5 min). The post-nuclear supernatant was centrifuged at 100 000 rpm ($350\,000\times g$) for 10 min in a Beckman TL-100 tabletop centrifuge. Aliquots (50 μl) of the supernatants (cytosol) were loaded on S200 gel filtration column (Superdex-200 PC 3.2/30) mounted in a SMART-system (Pharmacia) equilibrated in RB containing 1 mM DTT and 10% ethyleneglycol. Fractions (25 μl) were collected and analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis as described below. The elution profile of the gel filtration column was size calibrated using the set of protein markers (Sigma), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (160 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

2.4. Electrophoresis and immunoblot analysis

Aliquots from the S200 column were mixed with sample buffer containing DTT (100 mM final concentration) and boiled for 5 min. Proteins were separated on 7.5% SDS–polyacrylamide gels and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% non-fat milk in TBS containing 0.1% Triton X-100. Proteins were decorated with indicated antibodies followed by horseradish peroxidase conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Pierce).

2.5. Phagocytosis and fluorescence microscopy

Complement opsonized zymosan was prepared in human AB serum for 30 min as previously described [20]. IgG particles were made by incubation of zymosan (Sigma) with rabbit anti-zymosan IgG as directed by the manufacturer (Molecular Probes). Opsonized particles were washed three times with PBS and resuspended in RPMI–HEPES (RPMI-1640 buffered with 20 mM Na^+ HEPES, pH 7.3).

J774A.1 cells were plated in culture medium on glass coverslips. For the activation of complement receptors the cells were plated on coverslips pretreated with fibronectin (100 μg/ml) for 30 min at 37°C. After 2 h of incubation non-adherent cells were removed by aspiration. Adherent cells were washed with RPMI–HEPES, then 0.5 ml of medium containing 5–10 opsonized particles/cell was added and the zymosan was centrifuged onto macrophages at $450\times g$ for 2 min at room temperature. Ingestion was initiated by incubating the cells at 37°C. After various times cells were fixed with 3% paraformaldehyde in PBS for 30 min. The coverslips were washed with PBS and the cells permeabilized in acetone at −20°C for 30 s. Acetone was then removed by washing with PBS. Permeabilized cells were blocked with PBS containing 10% goat serum and 0.5% BSA for 15 min, followed by the incubation with the first antibody for 1 h in the same buffer. After washing, the secondary FITC-conjugated goat anti-rabbit IgG was added together with rhodamin–phalloidin.

3. Results and discussion

3.1. Constitutive activation of PI 3-kinase leads to dissociation of coronin from large cytosolic complexes

In order to investigate a potential role for PI 3-kinase for the assembly of cytosolic protein complexes we used parental GM-1 cells and GM-128 cells which express a constitutively active PI 3-kinase [19]. We compared the stability of oligomerized proteins composed of coronin and $p47^{phox}$ in the cytosol from both cell lines. To reduce basal activities cytosols were prepared from serum-starved GM-1 and GM-128 cells and fractionated on a Superdex 200 gel filtration column. The content of the p85 subunit of PI 3-kinase, coronin and $p47^{phox}$ in each fraction was examined by Western blot analysis. Fig. 1A shows a typical elution profile obtained with cytosols derived from GM-1 cells. The majority of p85 eluted with an apparent molecular mass of 200 kDa as expected for the p85/110 heterodimer [11]. The bulk of coronin (57 kDa) and $p47^{phox}$ co-eluted with a peak in fractions 12–14 corresponding to a size of approximately 170–200 kDa, much higher than their respective molecular masses. This finding is in agreement with the observations by Grogan et al. [5] who reported that in resting neutrophils coronin is associated with $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ forming a complex that elutes with an apparent mass of 200 kDa. In GM-1 cells and neutrophils (not shown) a somewhat smaller pool of coronin can be detected in early column fractions 5–8, that are devoid of $p47^{phox}$ and $p67^{phox}$ (not shown). The estimated molecular mass of the protein complexes eluting in fractions 5–8 is approximately 400–600 kDa. Grogan et al. [5] reported that in neutrophils coronin is far more abundant than the *phox* proteins. The content of $p47^{phox}$ and $p67^{phox}$ in GM-1 cells is lower than in neutrophils whereas the level of coronin is comparable. Thus the presence of *phox* protein independent pools of coronin are conceivable, however the size of the complex was not expected. In contrast to the parental cells the high molecular weight pool of coronin is absent in GM-128 cells

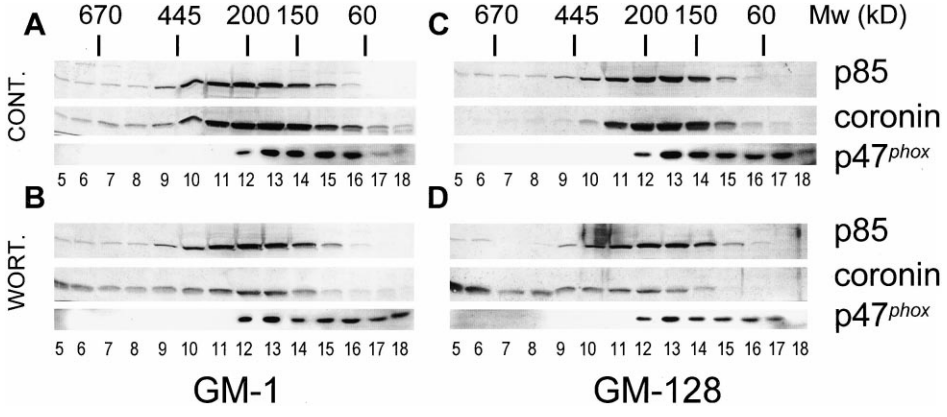

Fig. 1. Association of coronin with large cytosolic complexes is regulated by PI 3-kinase activity. Cytosols from parental GM-1 (A, B) and GM-128 cells, expressing constitutively active PI 3-kinase (C, D), were fractionated on a Superdex 200 gel filtration column. Column fractions were subjected to SDS–PAGE and the elution profiles of p85, coronin and $p47^{phox}$ were determined by Western blot analysis. C: Constitutive PI 3-kinase activity causes the dissociation of coronin from large cytosolic complexes. B and D: Inhibition of PI 3-kinase with 100 nM wortmannin leads to accumulation of coronin in high molecular weight fractions. Distribution of p85 and $p47^{phox}$ was the same in all cytosols (A–D). The elution of the molecular weight markers (M_w) is indicated above. The fraction numbers are indicated at the bottom.

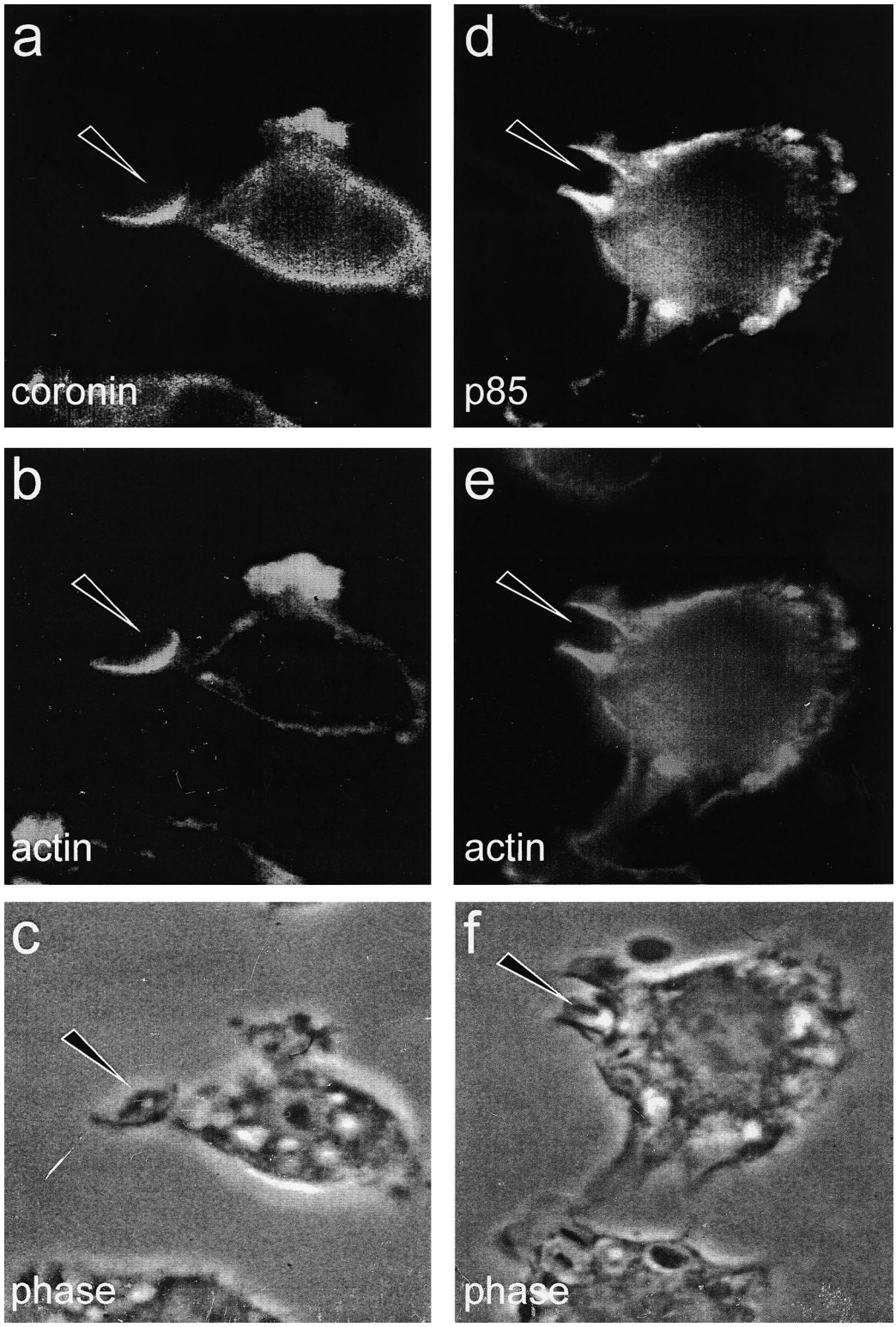

Fig. 2. Association of coronin and PI 3-kinase with phagocytic cups and nascent phagosomes, but not with maturing phagosomes, during complement receptor mediated phagocytosis. J774A.1 macrophages plated on glass coverslips were exposed for 30 s (a–f), 3 min (g–l) and 60 min (m–r) to complement opsonized zymosan particles at 37°C. Cells were fixed with paraformaldehyde, permeabilized and double stained for F-actin (b, e, h, k, n and q) and p85 (d, j and p) or coronin (a, g and m), as described in Section 2. Panels c, f, i, l, o and r are the corresponding phase images. Open arrows show phagocytic cups, white arrows indicate early phagosomes, and black arrows denote mature phagosomes.

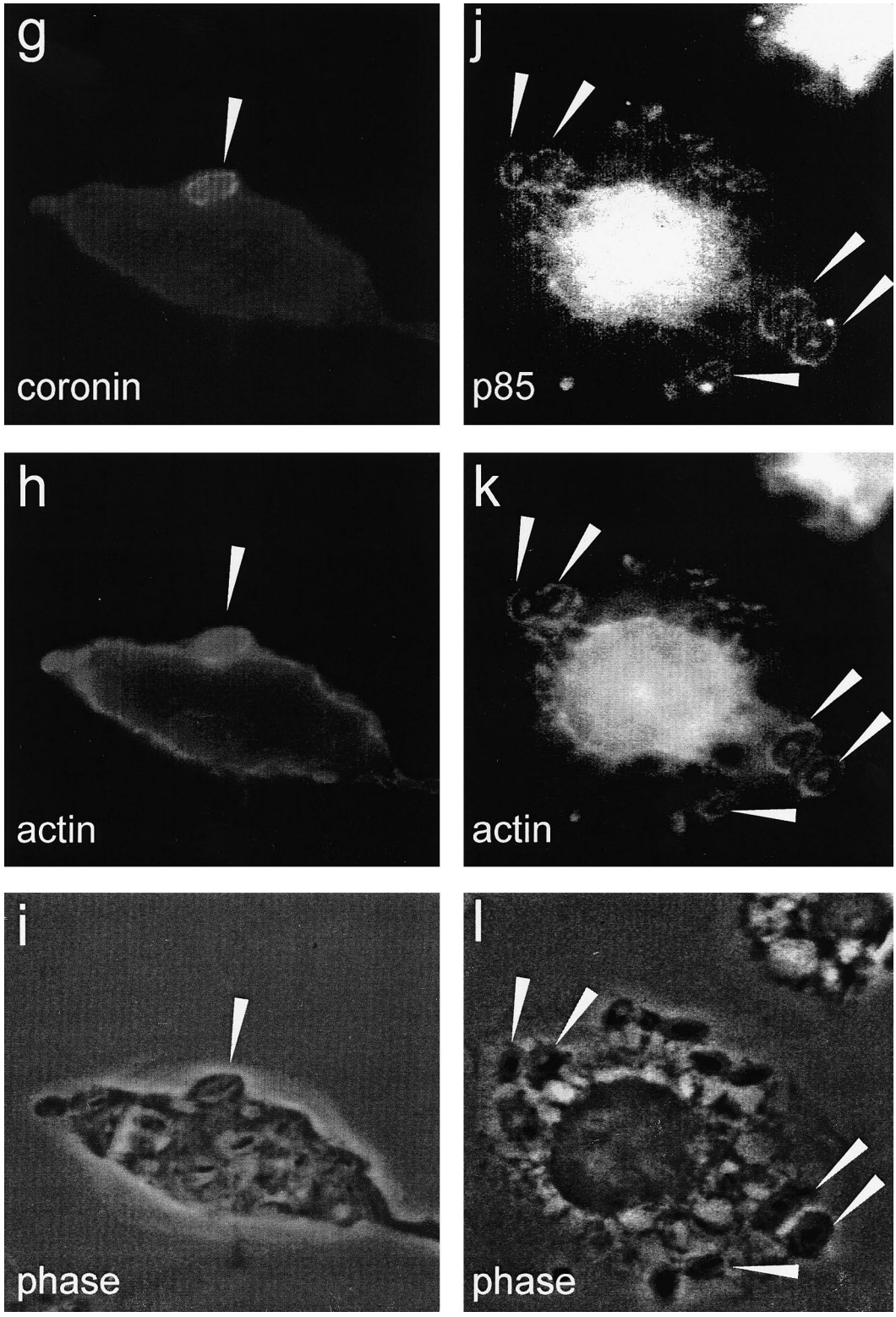

Fig. 2 (*continued*).

(Fig. 1C) whereas the amount of coronin in fractions 12–14 appears not to be altered. This finding implies that activation of PI 3-kinase causes the solubilization of the high molecular weight coronin complexes. To confirm the role of PI 3-kinase we tested the effect of wortmannin on the stability of the coronin complexes. Treatment of GM-128 cells for 30 min with 100 nM wortmannin, a concentration which specifically eliminates all PI 3-kinase activity, reverts the effect of the constitutively active enzyme (Fig. 1D). The inhibitor causes a marked increase of coronin that elutes with an apparent molecular mass of 400–600 kDa (fractions 5–8) (Fig. 1D). In the parental cells the effect of wortmannin is less striking,

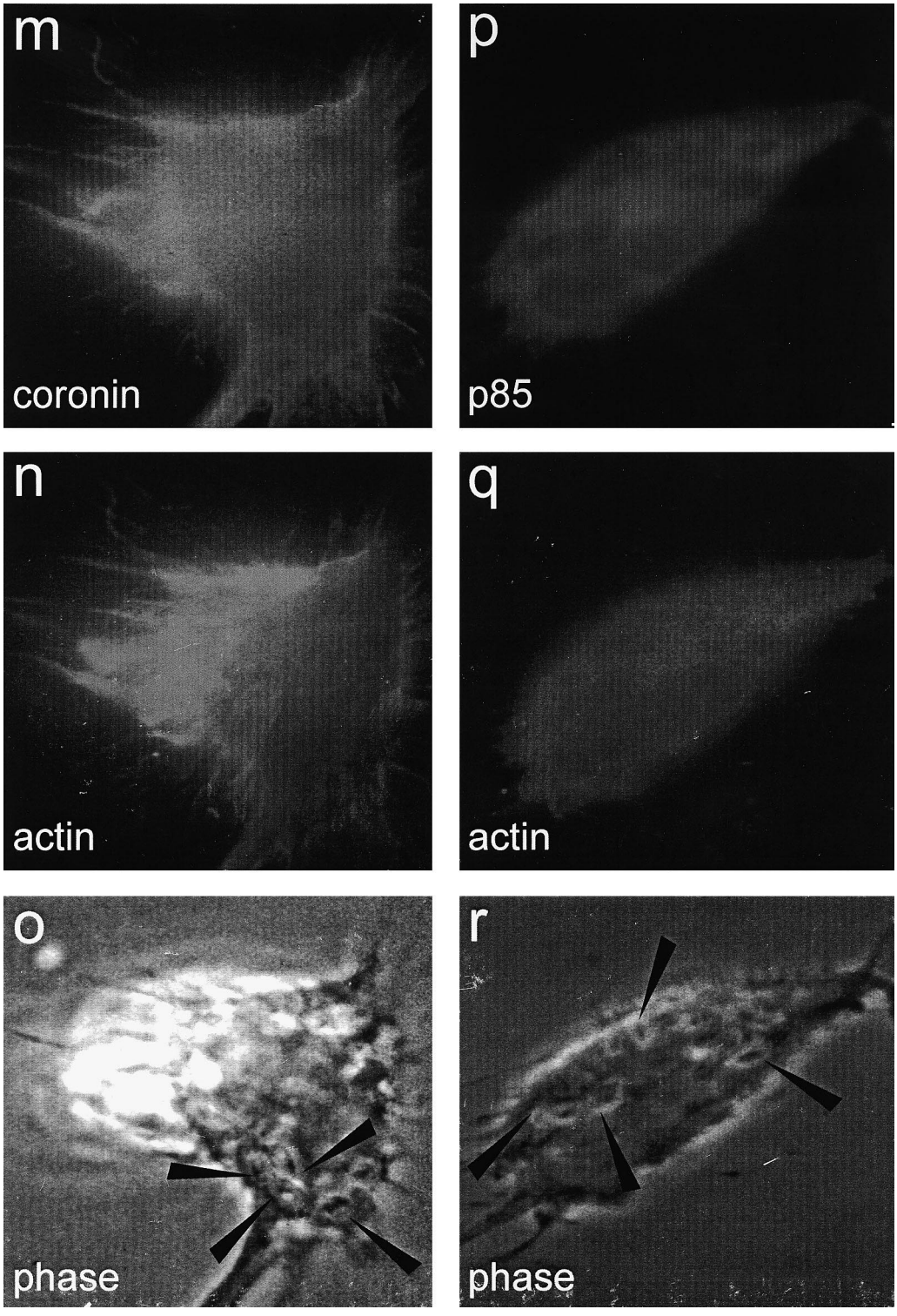

Fig. 2 (*continued*).

still a moderate increase of coronin in fraction 5–8 could be observed (Fig. 1B), confirming that PI 3-kinase activity destabilizes the protein complex. Constitutive PI 3-kinase activity or inhibition of PI 3-kinase with wortmannin does not change the elution profile of p85 or $p47^{phox}$ (Fig. 1), suggesting that the subcellular distribution of coronin is regulated by different signal transduction mechanisms. In addition, coronin did not co-precipitate with anti-p85 immunocomplexes, indicating that the protein does not directly interact with type I_A PI 3-kinases.

3.2. *PI 3-kinase and coronin participate in early stages of Fc- and complement receptor mediated phagocytosis*

Coronin is involved in the formation of surface projections of *D. discoideum* [2] and is found in the cortical skeleton of neutrophil and macrophage phagosomes [3,5]. We therefore

investigated the engagement of coronin and PI 3-kinase in phagocytosis of macrophages. For the studies, the murine macrophage cell line J774A.1 were chosen, because the cells grow adherent and are more suitable for immunofluorescence studies. Initial studies were performed with GM-1 cells and gave similar results. Fc- and complement receptor mediated phagocytosis was stimulated with IgG or complement opsonized zymosan. Opsonins were centrifuged onto serum-starved J774A.1 cells to initiate phagocytosis and then incubated at 37°C to allow phagosome formation and maturation. Samples were taken at indicated times, the cells fixed with paraformaldehyde and processed for indirect immunofluorescence analysis. Coronin and PI 3-kinase were found to associate with cup-like structures of nascent phagosomes (Fig. 2a,d) where they co-localize with F-actin at the phagosome membrane (Fig. 2b,e). After 1 min at 37°C complement opsonized particles were fully ingested as shown by circular rims of rhodamin–phalloidin stained F-actin that surrounded the newly formed phagosomes (Fig. 2h,k). Both coronin and PI 3-kinase co-localize with the actin skeleton at the phagosomes (Fig. 2g,j). Like F-actin the proteins remain associated with the phagosomes for up to 20 min (data not shown). Maturation to phagolysosomes was shown to be accompanied by the loss of the F-actin coat [21]. Along with it (Fig. 2n,q) we show here the loss of coronin and PI 3-kinase (Fig. 2m,p). Similar observations were made when the cells were exposed to IgG opsonized zymosan (data not shown). The results are in agreement with a previous report by Allen et al. [7] showing that coronin is shedded from nascent phagosomes once the particles are internalized. Furthermore evidence was presented that the loss of the coronin coat is essential for fusion of the phagosomes with lysosomes [6]. The association of p85 with early phagosomes has been described reported [14,17]. However, Strzelecka et al. [22] could not detect the recruitment of p85 in Fc-receptor mediated phagocytosis of thioglycollate activated macrophages. The discrepancy with the present data could arise from the use of antibodies with lower affinity for p85.

We found using gel filtration that in neutrophils and GM-1 cells coronin resides in resting cells in at least two distinct pools: a high molecular weight 400–600 kDa pool that is solubilized by PI 3-kinase activity and a 200 kDa pool that appears to be associated with *phox* components and is insensitive to PI 3-kinase activity. In line with this we observed, using immunofluorescence analysis, that coronin and PI 3-kinase co-localize at the phagosome. This finding is in agreement with a previous report by Allen et al. presenting evidence that the temporal association of $p47^{phox}$ and $p67^{phox}$ with phagosomes is not correlated with the association of coronin [7]. By contrast, Grogan et al. [5] reported that in neutrophils from patients with $p47^{phox}$ or $p67^{phox}$ deficiency coronin is not redistributed upon stimulation with PMA. However, phorbol ester treatment of phagocytes does not lead to PI 3-kinase activation and subsequent PIP_3 formation. Taken together it appears that coronin is recruited to the phagosomes from a high molecular weight complex that is independent of $p47^{phox}$ and $p67^{phox}$ and the association of coronin is independent of the activation of the NADPH oxidase. The conclusion is in agreement with the findings in *D. disciodeum* which do not possess an NADPH oxidase. Thus, our results suggest that the pool of coronin that is involved in phagocytosis is regulated by PI 3-kinase.

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