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Localization of five annexins in J774 macrophages and on isolated phagosomes

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SUMMARY

Annexins are a family of structurally related proteins which bind phospholipids in a calcium-dependent manner. Although the precise functions of annexins are unknown, there is an accumulating set of data arguing for a role for some of them in vesicular transport and, specifically, in membrane-membrane or membrane-cytoskeletal interactions during these processes. Here we describe our qualitative and quantitative analysis of the localization of annexins I-V in J774 macrophages that had internalized latex beads, both with and without IgG opsonization. Our results show that whereas all these annexins are present on both the plasma membrane and on phagosomes, the localization on other organelles differs. Annexins I, II, III and V were detected on early endosomes, while only annexin V was seen on late endocytic organelles and mitochondria. Annexins I and II distributed along the plasma membrane non-uniformly and co-localized with F-actin at the sites of membrane protrusions. We also investigated by western blot analysis the association of annexins with purified phagosomes isolated at different time-points after latex bead internalization. While the amounts of annexins I, II, III and V associated with phagosomes were similar at all times after their formation, the level of annexin IV was significantly higher on older phagosomes. Whereas annexins I, II, IV and V could be removed from phagosome membranes with a Ca^{2+} chelator they remained membrane bound under low calcium conditions. In contrast, annexin III was removed under these conditions and needed a relatively high Ca^{2+} concentration to remain phagosome bound. Because of their purity and ease of preparation we suggest that phagosomes are a powerful system to study the potential role of annexins in membrane traffic.

Key words: Annexin, Mouse, Macrophage, Endosome, Phagosome, ImmunoEM

INTRODUCTION

The annexin family of proteins presently consists of at least 20 members expressed in organisms as widely diverse as higher plants, slime moulds, metazoans, insects, birds and mammals (Morgan and Fernandes, 1995). For a protein to qualify as a member of this family, it must satisfy two essential criteria, one structural and one biochemical. These are, first, the presence of a conserved 70 amino acid sequence, repeated either four or eight (annexin VI) times in the overall structure and, second, the ability to bind phospholipids in a calcium-dependent manner (Creutz, 1992; Moss, 1992; Raynal and Pollard, 1994). The diverse biological activities of the annexins appear to depend on the NH_2-terminal tail which is variable in both length and sequence. The accumulation of structural and biochemical data on the annexins has progressed far more rapidly than our understanding of their function.

The precise roles of annexins are indeed far from understood but they have been suggested to function in a broad range of intracellular processes, including signal transduction, DNA replication, cell transformation, ion-channel formation and apoptosis (Moss, 1992; Gruenberg and Emans, 1993; Raynal and Pollard, 1994). While the latter functions remain controversial there is an accumulating set of data arguing more strongly for a role of at least some of the annexins in vesicular transport and, specifically, in membrane-membrane or membrane-cytoskeletal interactions during these processes.

During the process of exocytosis it has been argued that certain members of this protein family may initiate contact and perhaps even fusion between a secretory vesicle membrane and the plasma membrane (for review see Creutz, 1992; Raynal and Pollard, 1994). Annexin XIIIb, for example, was recently identified as a component of TGN-derived vesicles destined for the apical membrane of MDCK cells (Fielder et al., 1995). Also along the endocytic pathway numerous observations have been reported implicating a role for the annexins

(for review see Gruenberg and Emans, 1993; Burgoyne and Clague, 1994; Raynal and Pollard, 1994). While data relating annexin VI to coated-pit budding are controversial (Lin et al., 1992; Smythe et al., 1994), this protein was found in association with endosomal fractions (Jackle et al., 1994). There is also growing evidence that annexin II is involved in some endosome functions; it is suggested to be involved in a docking and/or fusion process between early endosomes from in vitro and in vivo studies (Emans et al., 1993; Mayorga et al., 1994; Harder and Gerke, 1993). Annexin I has also been localized to early endosomes. In this case the association with this compartment has been shown to require the N-terminal end of the molecule and to depend on calcium (Seemann et al., 1996). Annexin I has additionally been found on multivesicular bodies and it has been proposed that phosphorylation of this annexin is necessary for the sorting of EGF-receptor from early endosomes towards the lysosomes (Futter et al., 1993).

Annexins have also been found along the phagocytic pathway. Thus, during phagocytic uptake of opsonized yeast by neutrophils annexin III becomes translocated from a general cytoplasmic distribution to the close vicinity of phagosomes (Ernst, 1991). Furthermore, colocalization of annexins III, IV and V, but not I and VI was seen with chlamydial inclusions (phagosome-like structures) following the entry of *Chlamidia trachomatis* in to HeLa and McCoy cells (Majeed et al., 1994). Moreover, annexin III is associated with cytoplasmic granules in resting neutrophils and monocytes but is translocated to the plasma membrane and/or the phagosomal membrane following activation with phorbol esters or opsonized zymosan (Le Cabec and Maridonneau-Parini, 1994). Also in neutrophil fractions a similar translocation of annexin I to phagosomes was seen upon stimulation by opsonized zymosan, but not in the presence of phorbol ester, using immunoblotting analysis (Kaufman et al., 1996). Further, upon phagocytosis of yeast, *Escherichia coli* or killed *Brucella suis*, annexin I rapidly translocated to, and concentrated around, phagosomes, but in the case of live *Brucellae* this phenomenon was not observed (Harricane et al., 1996). A recent study by Collins et al. (1997) has also shown that phagosomes containing live *Leishmania mexicana* acquire annexin I.

In the present study we focussed on the detailed localization of annexins I-V, for which monospecific antibodies were available, in one cell type, the J774 mouse macrophage cell line. In this cell we have long been interested in the process of phagocytosis and its relation to the endocytic pathway. We have established an approach using 1 μm latex beads as phagocytic markers that facilitate the purification of phagosomes (or phagolysosomes) at any time after bead internalization (Desjardins et al., 1994a). These phagosomes undergo a complex series of biochemical changes as they age in the cell (for reviews see Desjardins, 1995; Griffiths, 1996). These maturation changes are correlated with a difference in their fusion capacity with endocytic organelles (A. Jahraus et al., unpublished), as well as their interactions with microtubules (Blocker et al., 1996, 1997). Two annexins had already been investigated in this system by western blot analysis: whereas annexin II was one of the very few proteins we have found to be at a constant level on phagosomes at all times up to 24 hours after latex bead internalization, annexin VI was significantly more concentrated on 24 hour phagosomes than on 2 hour ones (Desjardins et al., 1994b).

Here we describe our qualitative and quantitative immunocytochemical analysis of the localization of annexins I-V in J774 macrophages that had internalized latex beads. Our results show that whereas all these annexins are present on both the plasma membrane and on phagosomes, the localization on other organelles differs. While annexins I, II, III and V were detected on early endosomes, only annexin V was seen in significant amounts on late endosomes and mitochondria. We also investigated by western blot analysis the association of annexins with phagosomes isolated at different time-points after latex beads internalization. Whereas the amounts of annexins I, II, III and V associated with phagosomes were similar at all times, the level of annexin IV was higher on phagosomes at later times after their formation. We also describe the differential effect of Ca^{2+} on the association of these different annexins with phagosome membranes.

MATERIALS AND METHODS

Cell culture

J774 mouse macrophages were grown in 10 cm dishes in DME supplemented with 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

Phagosome formation and cell fractionation

Phagosomes were formed by the internalization into macrophages of latex beads (1 μm diameter, 10% suspension, Seradyn, Indianapolis, USA) diluted 1:200 in culture medium at 37°C. Macrophages were exposed to latex beads for a 20 minute pulse followed by a chase period without beads for 10 minutes as the earliest time point and a 1 hour pulse and 24 hour chase as the latest time point for the preparation of the oldest phagosomes. Internalization and chase times varied depending on the experiments, as mentioned in the figure legends. All pulses and chases were performed at 5% CO_2. Isolation of phagosomes was performed as described previously (Desjardins, 1994a). Briefly, after latex beads internalization, the cells were washed in cold phosphate-buffered saline (PBS) (3× 2 minutes) and scraped with a rubber policeman in PBS at 4°C. The cells were pelleted in homogenization buffer (HB: 250 mM sucrose, 3 mM imidazole, pH 7.4) at 4°C, resuspended in 1 ml of HB with protease inhibitors, and homogenized on ice using passage through a 22 gauge needle. In some experiments, all solutions were supplemented with 0.5 mM EDTA or 100 μM $CaCl_2$. The homogenization was carried out until about 90% of cells were broken without breakage of the nucleus, as monitored by light microscopy. Unbroken cells were pelleted in a 15 ml Falcon tube at 1,400 rpm for 10 minutes at 4°C and the supernatant, containing the phagosomes, was recovered. The phagosomes were then isolated by flotation on a sucrose step gradient (all sucrose solution were w/w in 3 mM imidazole, pH 7.4) as follows. The supernatant was brought to 40% sucrose by adding the same volume of a 62% sucrose solution. Then 25% sucrose and 10% sucrose solutions were layered sequentially on top of the 40% sucrose. Centrifugation was done in an SW40 rotor (Beckman Instruments, Palo Alto, CA) for 1 hour at 100,000 ***g*** at 4°C. The phagosome band was collected from the interface of the 10 and 25% sucrose solution. The phagosomes were pelleted by a 15 minute centrifugation at 40,000 ***g*** in an SW40 rotor at 4°C.

Opsonization of latex beads

Carboxylate-modified latex beads (CML, 1 or 5 μm diameter, Seradyn) were derivitized with dinitrophenyl (DNP). IgG-coated

beads were obtained by incubation of DNP-coated beads with 25 μg/ml affinity-purified rat anti-DNP IgG (Biosys, France). The beads were then washed three times and suspended in PBS (Aderem et al., 1985). Immunoelectron microscopy using whole mounts and negative staining after labeling with polyclonal anti-rat antibody and Protein A-gold demonstrated that IgG was uniformly distributed on the surface of beads.

Immunoblots

The kinetic analysis of annexins association with phagosomes was performed using western blot analysis. For each time point, the proteins from the same number of phagosomes (determined by forward light angle scattering analysis in a flow cytometer) were lysed in Laemmli buffer and separated by 12% SDS-PAGE. Proteins were then transferred onto nitrocellulose filters using standard procedures. All the antibodies used for western blotting and for immunocytochemistry have been previously shown to be monospecific for the annexin of interest. Annexin I was revealed using affinity purified rabbit polyclonal antibodies (Harricane et al., 1996) while annexin II was identified using a mouse monoclonal antibody (Osborn et al., 1988). The monospecific antiserum to annexin III (Ernst et al., 1990; Ernst, 1991) and the affinity purified rabbit polyclonal antibodies to annexin IV (Gerke and Weber, 1984) and annexin V (Jans et al., 1995) have also been described. The reactions were developed using enhanced chemiluminescence.

Immunocytochemistry on cryosections

Cells were simultaneously loaded with endocytic and phagocytic markers as described previously (Rabinowitz et al., 1992). To label late endocytic compartments, a BSA-16 nm gold conjugate (A_{520} = 0.5) was added to the cell for 1 hour and after washing cells were cultivated overnight. To form phagosomes the sterile latex beads (1 μm diameter, 10% suspension, Seradyn, Indianapolis, USA) diluted 1:200 in culture medium were added for 1 hour (pulse period) and after washing cultured for another hour (chase period). The early endosome compartment was loaded with a BSA-5 nm gold conjugate (A_{520} = 10) for 5 minutes. The cell monolayer was washed twice with ice-cold PBS and treated with 50 mg/ml proteinase K (Green et al., 1981) in PBS for 2-3 minutes on ice. For all the annexins preliminary experiments were also carried out on cells that were fixed and scraped, with no obvious difference evident. Following the removal of the cells from the plastic support a fixative (8% paraformaldehyde in 250 mM Hepes, pH 7.4) was placed in a 1.5 ml Eppendorf tube on ice and the proteinase K-cell mixture was layered on the fixative. This regime helps to stabilize the cells against the subsequent centrifugation (3,000 rpm, 1 minute). The supernatant was removed without disturbing the pellet and fresh fixative at room temperature was added for 30 minutes followed by centrifugation at top speed in a bench-top centrifuge. Subsequently the cells were infiltrated with 2.1 M sucrose overnight at 4°C and frozen in liquid nitrogen. Cryosectioning and labeling with antibodies followed by Protein A-gold were carried out, as described previously (Griffiths, 1993).

For labeling the plasma membrane of J774 cells, we used biotinylated lectin from peanut (Boehringer Mannheim, Germany). For immunoelectronmicroscopy study thin sections were labeled with this reagent for 20 minutes, followed by monoclonal anti-biotin antibody (Boehringer Mannheim, Germany), rabbit anti-mouse antibody (Cappel Laboratories, Cochranville, PA) and Protein A-gold conjugate (purchased from the Dept of Cell Biology, University of Utrecht).

Quantitation

After fixation and cryosectioning the sections were labeled with annexin antibodies and Protein A-gold (10 nm). In the case of monoclonal anti-annexin II antibody we used an additional step with a rabbit anti-mouse antibody. Twenty micrographs of each cell structure of interest were taken in a systematic fashion at a primary magnification of ×25,000 by moving the grid systematically from one structure to the next using the translation controls of the electron microscope. These images were enlarged 4.09× on an EMBL-made projector system. The number of gold particles labeling a membrane profile were related to the length of that profile by intersection counting, as described by Griffiths (1993).

Relative surface areas

For estimating the relative surface areas of phagosomes, early endosomes and plasma membrane we used the ratio of intersections between these organelles and a test line lattice grid system (spacing between lines was 20 mm). For this, systematic random micrographs of sections were taken at a primary magnification of ×25,000, which were then enlarged 4.09×. The test line system was overlaid on the micrographs and the number of intersections was counted with the organelle of interest. The ratio of intersections between the different organelles is an unbiased estimation of their relative surface areas (see Griffiths, 1993).

Immunofluorescence

Cells grown on glass coverslips were fixed with 3.7% formaldehyde in PBS for 10 minutes, and subsequently permeabilized with 0.1% Triton X-100 in PBS for 1 minute. Unspecific staining was reduced by incubation with 1% bovine serum albumin (BSA). All incubations with phalloidin-rhodamine and anti-annexin I or anti-annexin II antibodies were carried out for 30 minutes at room temperature. To trigger phagocytosis for the light microscopy experiments either the sterile latex beads (5 μm diameter, 5% suspension, Seradyn, Indianapolis, USA) diluted 1:5,000 in culture medium or IgG-coated beads the same concentration were added to the monolayers of J774 cells for 1 hour (pulse period) and after washing cultured 1 hour more (chase period). Cells were fixed in methanol at −20°C, washed three times in PBS containing 1% BSA and incubated with 4% normal calf serum for 30 minutes at room temperature (Majeed et al., 1994). Next anti-annexin antibodies were added and visualized using secondary rhodamine-coupled goat anti-rabbit antibody or anti-mouse antibody (Cappel Laboratories, Cochranville, PA).

For lectin staining the cells were fixed with 3.7% formaldehyde in PBS for 10 minutes, incubated with 20 mg/ml biotinylated agglutinin from peanut (Boehringer Mannheim, Germany) for 20 minutes, followed by monoclonal anti-biotin antibody (Bohringer Mannheim, Germany) and rhodamine-coupled rabbit anti-mouse antibody (Cappel Laboratories, Cochranville,PA).

Confocal images of J774 cells were taken using the EMBL confocal microscope.

RESULTS

For all the immunoelectronmicroscopy studies we used J774 mouse macrophages simultaneously loaded with endocytic and phagocytic markers. To label late endocytic compartments, a 16 nm gold-bovine serum albumin conjugate was added to the cell for 1 hour and, after washing, the cells were cultured overnight in the absence of gold. This marker labels late endocytic organelles. In general, the bulk of this gold accumulates in mostly spherical vesicles that we classify as lysosomes with significantly less and more dispersed gold particles also remaining in the larger, more heterogeneous structures we consider to be late endosomes. In this study we made no attempt to distinguish these two sets of endocytic organelles. Subsequently, 1 μm latex beads were incubated for a 1 hour pulse followed by a 1 hour chase period in the absence of beads to form ‘2 hour’ phagosomes. Finally, BSA-5 nm gold was internalized for the last 5 minutes before fixation to mark

early endosomes. The cells were then prepared for cryosectioning and labeling using previously characterized monospecific antibodies against annexins I-V (see Material and Methods). When specimens were labeled with well characterized but irrelevant antibodies against vaccinia virus antigens there were never more than a few gold particles seen over profiles of whole cells. (A quantitation of these antibodies will be shown below.) All five annexins were also localized by immunofluorescence microscopy. Only the significant observations with this approach are mentioned below.

Annexin I

Using affinity purified anti-annexin I antibodies we obtained significant labeling of the cytoplasmic surface of the plasma membrane and early endosomes (Fig. 1). We could also detect some labeling of coated pits and coated vesicles (many of the latter also contained 5 nm BSA-gold) (Fig. 1A) and, occasionally, the endoplasmic reticulum (ER) (Fig. 1B). The distribution of gold particles along the plasma membrane was non-uniform: stronger labeling was observed on the cell protrusions and microvilli as compared to 'smooth' parts of the plasma membrane (Fig. 2). This observation was confirmed by immunofluorescence microscopy: the sites of apparent cell ruffles showed a brighter fluorescent pattern (Fig. 3A). These parts of the cells were also labeled with phalloidin-rhodamine as a marker for F-actin. Confocal microscope images allowed us to see the sites of co-localization of annexin I and actin more distinctly (Fig. 4).

Using internalized latex beads as markers we could observe a significant level of anti-annexin I immunogold labeling on the phagosome membrane (Fig. 1B) in addition to the significant labeling of early endosomes identified by the presence of 5 nm BSA-gold. All other organelles were not convincingly labeled.

To document the gold labeling more rigorously we performed a quantitative analysis. A general problem we faced in the quantitative analysis of the annexins in this paper was the following. Normally when we quantify membrane antigens in the biosynthetic or endocytic pathway we refer the specific

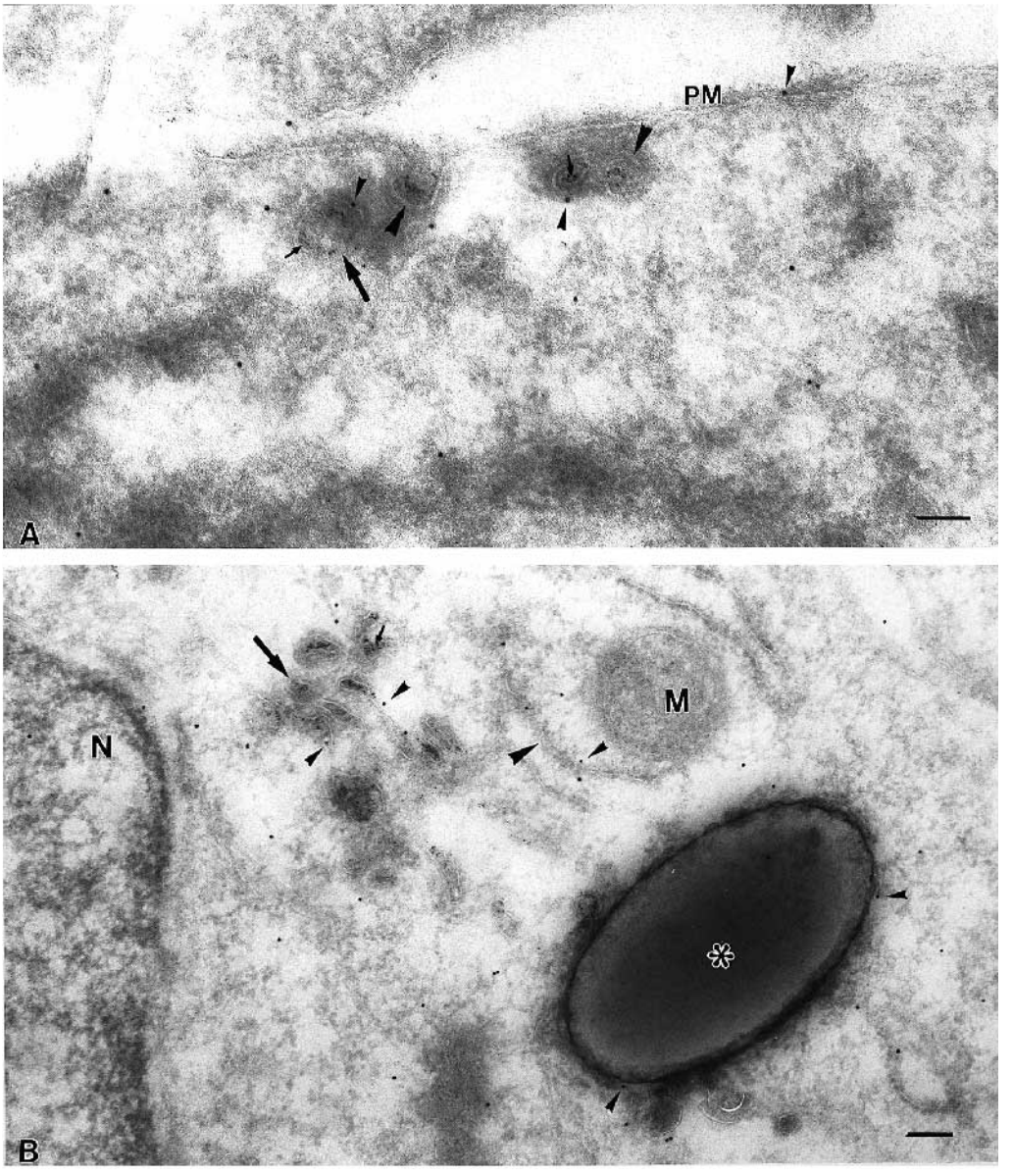

Fig. 1. Immunolocalization of annexin I in J774 cells. In all EM figures BSA-16 nm gold was used to label late endocytic structure and 1 μm latex beads were used to label phagosomes. The early endosome compartment was loaded with a BSA-5 nm gold conjugate (small arrows) for 5 minutes. In this figure cryosections were labeled with affinity purified anti-annexin I antibody followed by 10 nm Protein A-gold. This annexin (small arrowheads) was localized to the plasma membrane (PM), clathrin-coated pits and vesicles (A, large arrowheads), early endosomes (large arrows) and endoplasmic reticulum (B, large arrowheads). In B, specific labeling is associated with the early endosomes (large arrows) and phagosomes (asterisk). M, mitochondria; N, nucleus. Bars, 100 nm.

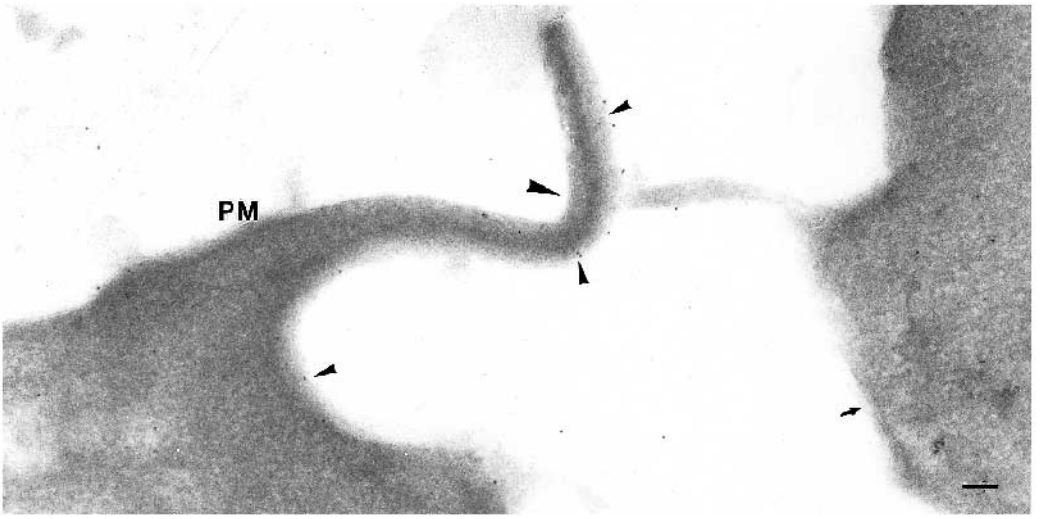

Fig. 2. Localization of annexin I to the plasma membrane. Distribution of annexin I (small arrowheads) along the plasma membrane (PM) is non-uniform: a microvillus (large arrowhead) is labeled stronger than the 'smooth' parts of plasma membrane (small arrow). Bar, 100 nm.

level of labeling (the signal) to an organelle such as the mitochondria or the nuclear matrix, organelles which one can confidently expect not to contain the antigen (therefore a reflection of background labeling or 'noise') (see Griffiths, 1993). In the case of the annexins, however, there was no intracellular membrane organelle which one could confidently predict to be free of antigen. Our rationale for overcoming this problem was to quantify the level of labeling on those organelles which consistently appeared to label in a systematic evaluation of the sections and to compare this level with the labeling obtained with antibodies against irrelevant viral antigens (background). We arbitrarily decided to consider those organelles to be specifically labeled with an annexin antibody only when the level of labeling was at least three times higher than the level of back-

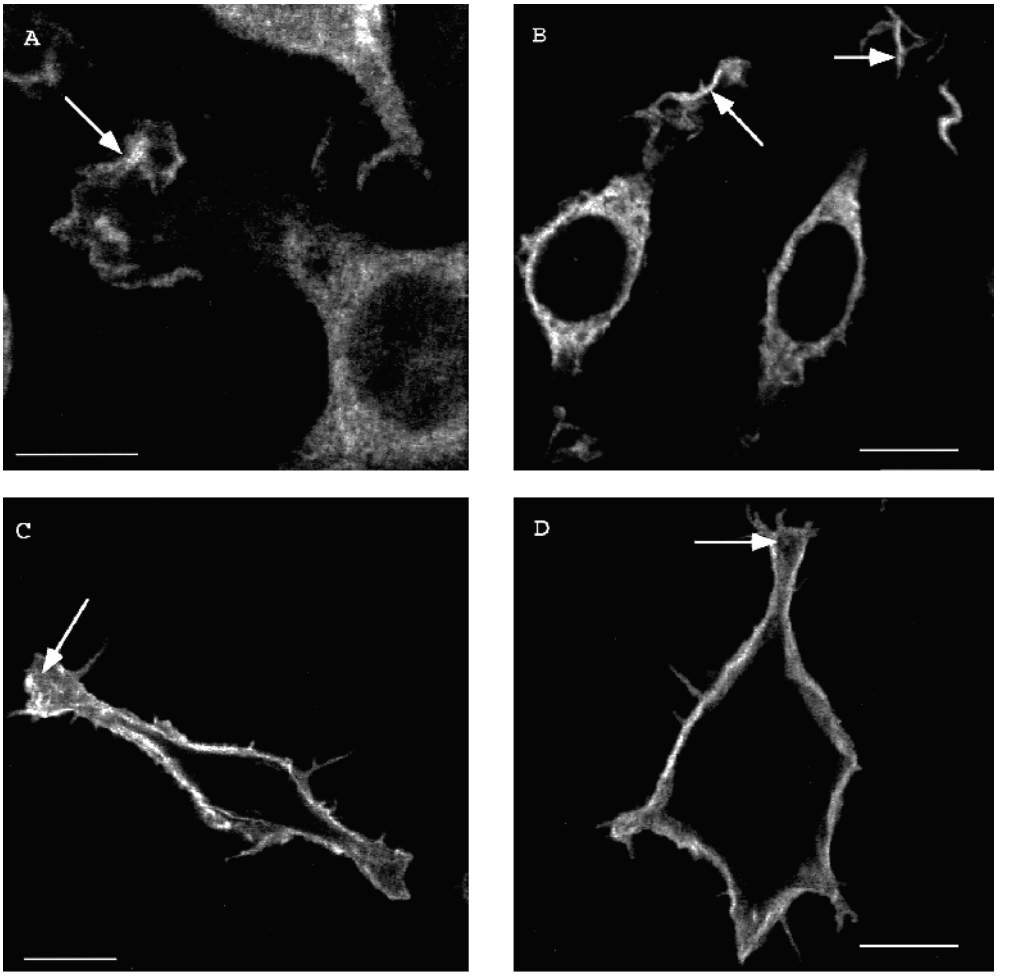

Fig. 3. Immunofluorescence labeling of annexin I and annexin II in J774 permeabilized cells. The strongest fluorescent pattern for annexin I (A) and for annexin II (B) was observed at the sites of cell ruffles (arrows) by confocal microscopy. In contrast the labeling with peanut agglutinin (C,D) is uniform over the entire plasma membrane. Bars, 10 μm.

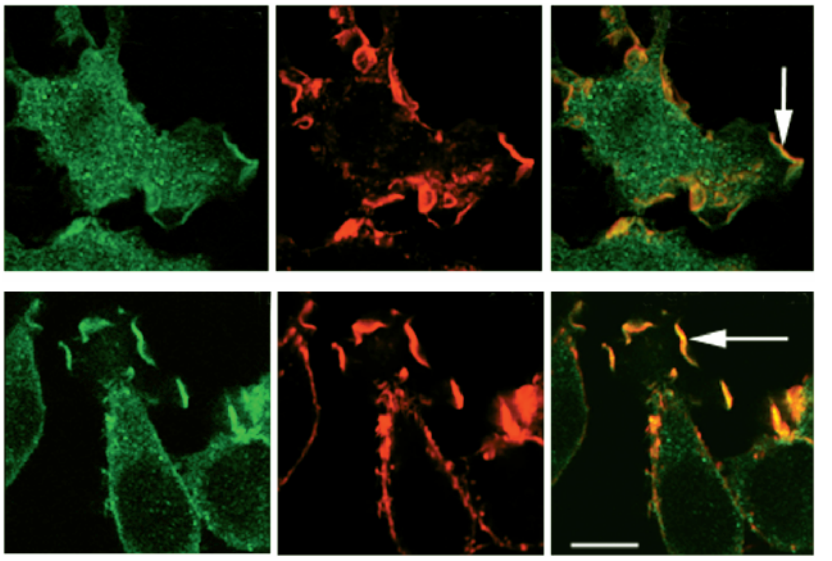

Fig. 4. Co-localization of annexin I and annexin II with F-actin in J774 cells. Cells were double labeled with phalloidin-rhodamine (red) and anti-annexin I and anti-annexin II antibodies (green) and visualized by confocal microscopy. Actin shows prominent co-localization with annexin I and annexin II at the sites of membrane ruffles (arrows). Bar 10 μm.

ground. We emphasize that this rationale does not allow us to unequivocally identify organelles that have low levels of annexins, just above background levels.

As shown in Fig. 9A, the highest density of anti-annexin I labeling was found on the early endosomes. Phagosomes also showed a significant, but lower level of labeling. The density of gold particles on cell protrusions was higher than on 'smooth' parts of the plasma membrane. We did not detect any significant labeling of late endosomes nor of mitochondria. Moreover, the sporadic labeling of the rough endoplasmic reticulum (Fig. 1B) was not significant in this analysis. The density of gold on clathrin-coated vesicles was similar to that seen on 'smooth' parts of the plasma membrane.

In order to determine whether the accumulation of annexin I on phagosomes was transient, we performed an immunoblotting analysis of phagosomal fractions purified at different stages of their maturation. Macrophages were exposed to latex beads for a 20 minute pulse followed by a chase period without beads for 10 minutes as the earliest time point and a 1 hour pulse and 24 hour chase as the latest time. Phagosomes were then isolated and phagosomal proteins subjected to immunoblotting with the anti-annexin I antibody. As shown in Fig. 10A annexin I was present in the phagosome fraction at similar levels at all stages of phagosome maturation tested. In some experiments at the latest time point (1 hour pulse and 24 hours chase) we supplemented all solutions with 0.5 mM EDTA (Seemann et al., 1996). In this case we could not detect the presence of annexin I in the phagosome fraction. It seems that the association of annexin I with phagosomes is Ca^{2+}-dependent, since this protein is released from phagosomal membranes by Ca^{2+} chelation.

Annexin II

For the localization of annexin II a monoclonal antibody was applied to thawed cryosections of J774 cells and visualized by a sandwich using rabbit anti-mouse antibodies and then Protein A-gold. Since one molecule of monoclonal antibody can bind more than one molecule of secondary rabbit anti-mouse antibody each of which is visualised by Protein A-gold, it results in clusters of 2-4 gold particles per antigen (see Griffiths, 1993).

There was significant labeling of the plasma membrane, as expected from earlier studies (Emans et al., 1993). The gold clusters along the plasma membrane were often distributed non-uniformly: while areas of cell protrusions, microvilli, and plasma membrane folds were strongly labeled, 'smoother' parts of the plasma membrane were more weakly labeled (Fig. 5A,B). This result was confirmed by immunofluorescence microscopy which demonstrated a strong fluorescence pattern at sites of cell microvilli\ruffles (Fig. 3B) and co-localization with F-actin at these sites (Fig. 4).

We considered the possibility that the appearance of a concentration of annexins I and II over the presumed ruffling regions was simply due to the fact that there is more membrane per unit volume in these regions. To control for this we used peanut lectin which can bind to surface galactose residues and which could be expected to label the entire plasma membrane in a uniform fashion. As shown in Fig. 3 application of this lectin, using an indirect labeling procedure, labeled the entire plasma membrane with no obvious concentration over ruffles (Fig. 3C,D).

By electron microsopy annexin II was found to be most abundant on the surface of early endosomes (Fig. 5A) as well as on the phagosome membrane (Fig. 5C).

For the quantitative analysis, as for annexin I, we distinguished between the 'smooth' part of the plasma membrane and those parts where protrusions occurred. As shown in Fig. 9B, the amount of gold particles per membrane length was almost three times higher for the areas of cell protuberanes relative to the smoother domains. In contrast the peanut lectin labeling, using an indirect gold approach, showed no significant difference between these two different parts of the plasma membrane (Fig. 9F), a relatively high density of gold particles was demonstrated for annexin II on early endosomes and phagosomes and a lower, but specific labeling was found on coated pits and vesicles. We did not detect significant labeling on late endocytic organelles, mitochondria or nuclear envelope (Fig. 9B).

Next, we investigated by western blot analysis the associa-

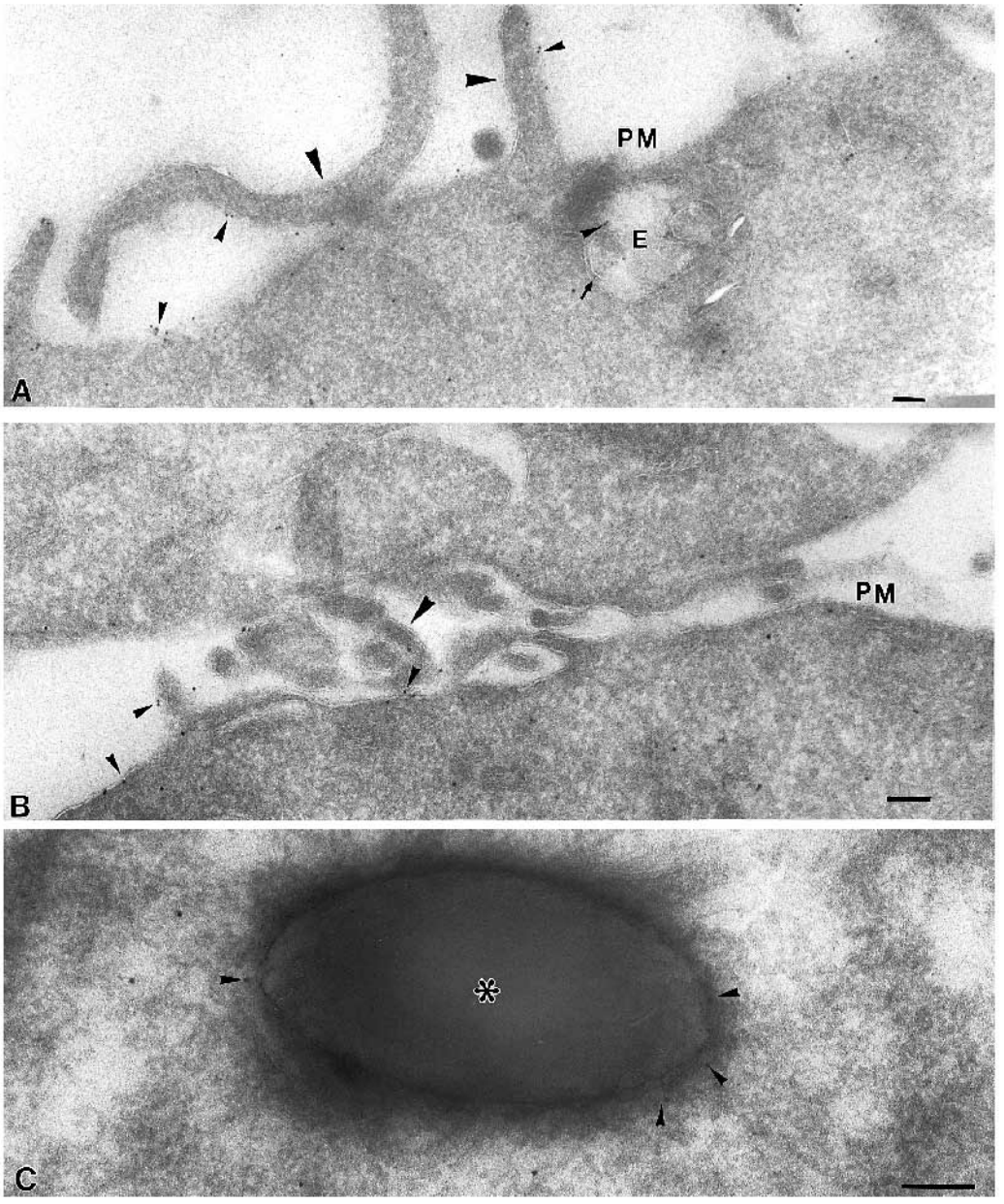

Fig. 5. Immunolocalization of annexin II in J774 cells. Cells were processed as described in Fig. 1, and a monoclonal antibody to annexin II was used for the localization of this protein (small arrowheads). Note the non-uniform distribution of gold particles along the plasma membrane (PM): cell microvilli and ruffles (A,B, large arrowheads) were labeled stronger than 'smooth' parts of the plasma membrane. Labeling of the early endosome compartment (E) containing a BSA-5 nm gold conjugate (small arrow) is shown in A. The membrane of the latex bead-containing phagosome (asterisk in C) is also specifically labeled by the anti-annexin II antibody. Bars, 100 nm.

tion of this protein with phagosomes relative to their age in the cell. As shown previously (Desjardins et al., 1994b), annexin II was present at a similar amount in all stages of phagosomes analysed (Fig. 10B). After the addition of 0.5 mM EDTA during phagosomes fractionation we could not detect this protein by western blotting indicating that the presence of annexin II in the phagosome fraction is Ca^{2+} dependent.

Annexin III

Using anti-annexin III antibodies on thawed cryosections of mouse macrophages J774 by systematic sampling of the grids we noticed significant labeling only of phagosomes and early endosomes at the electron microscope level (Fig. 6). A stereological analysis performed on electron micrographs confirmed these observations. As shown in Fig. 9C, the number of gold particles per length of early endosome was a little less than that seen for phagosomes but for both organelles the level was significantly higher than that seen over mitochondria or the nuclear envelope.

In initial western blotting attempts we failed to detect annexin on purified phagosomes when the preparation of the PNS and the gradient fractionation were performed in the absence of added Ca^{2+} (data not shown). In these experiments we found annexin III in the 40% sucrose layer (the load fraction) but not in the phagosome band at the 10%-25% sucrose interface. However, as shown in Fig. 10C annexin III was detected in the phagosome fraction when all solutions were supplemented with not less than 100 μM Ca^{2+}. At a still higher concentration (10 mM Ca^{2+}) the association of this annexin with the phagosome was lost (data not shown). These data indicate that the maintenance of the association of annexin III with the phagosome membrane depends on the presence of

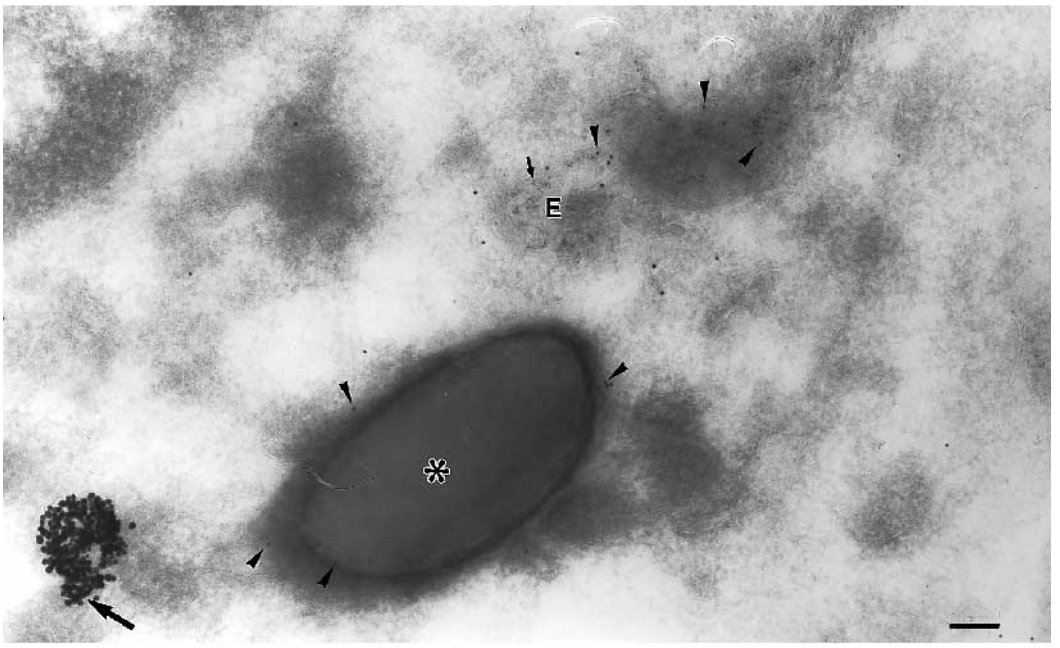

Fig. 6. Localization of annexin III to early endosomes and phagosomes. Annexin III (small arrowheads) was present on early endosomes (E) identified by the presence of 5 nm gold (small arrow) and phagosomes (asterisk), whereas no labeling was detected on a profile of a late endocytic element, probably a lysosome (large arrow). Bar, 100 nm.

micromolar concentrations of Ca^{2+}. Taking advantage of this fact we could then determine that the amount of annexin III bound to phagosomes did not change during their maturation (Fig. 10C).

Annexin IV

Using thawed cryosections we observed annexin IV labeling on the plasma membrane of the J774 macrophages, as well as on phagosomal membranes (Fig. 7A). Scattered labeling was also seen on early endosomes (Fig. 7B); however, our quantitative analysis showed significant labeling only of the plasma membrane and of phagosomes (Fig. 9D).

The kinetic association of annexin IV with phagosomes was studied by western blot analysis. As shown in Fig. 10D the amount of annexin IV was relatively low on the early phagosomes and increased in concentration on the phagosomes as they matured. Annexin IV was removed from phagosomes by the addition of EDTA.

Annexin V

When employing the annexin V antibody in our ultrastructural analysis we detected cytoplasmic labeling that was not apparently associated with any identifiable structures and was presumably cytoplasmic (not shown). We also saw significant labeling of the membranes of early endosomes, phagosomes, the plasma membrane as well as late endocytic organelles and mitochondria (Fig. 8).

Our quantitative analysis showed a relatively high density of gold particles on the plasma membrane, early endosomes, late endocytic organelles as well as phagosomes (Fig. 9E). For mitochondria the labeling obtained with anti-annexin V was three times higher than that seen with an additional control anti-mouse hepatitis virus M protein antibody (Fig. 9E). We also performed anti-annexin V immunogold labeling on thawed cryosections of NRK cells as a further control for the mitochondrial labeling. As shown in Fig. 9E the density of gold particles for mitochondria of NRK cells (Fig. 9E, mN) was also three times higher than that seen over the nuclear envelope of these cells (Fig. 9E, nN). We conclude that annexin V is indeed present on the cytoplasmic side of mitochondria. Binding of annexin V to mitochondrial membranes and mitoplasts from rat liver has been demonstrated previously by in vitro studies (Megli et al., 1995).

Western blot analysis of purified phagosomes showed that the amount of annexin V bound to phagosomes did not change during their maturation (Fig. 10E). Similar to annexins I, II and IV, the association of annexin V with the phagosomal membranes was abolished by Ca^{2+} chelation (Fig. 10E).

Estimation of total annexin labeling per cell

The quantitative estimates of the annexin labeling showed that all the annexins were enriched on the plasma membrane, the early endosomes (except annexin IV) and phagosomes. The parameter we estimated in that analysis was the number of gold particles per linear length of membrane profile which is directly proportional to membrane surface area (Griffiths, 1993). In order to relate these values to the total amount of annexins per organelle per cell we estimated the relative amount of membrane surface area in these three major organelles. For this, a transparent lattice grid test line system was placed on the projection of the negatives and the relative number of intersections between the test lines and either the plasma membrane, the early endosomes or phagosomes were classified. The resulting intersection counts gave a direct estimate of the relative amount of membrane in each organelle. The value we obtained was 1:2.1:4.3 for the ratio early endosomes:phagosomes:the plasma membrane per cell. Using these values we could then normalize the gold concentrations to give arbitrary numbers which are unbiased reflections of the total amount of membrane bound antigen per cell. Fig. 11 shows this for all the annexins. This analysis shows that for all annexins the majority of the label on a per cell basis is associated with the plasma membrane. However, the amount of label

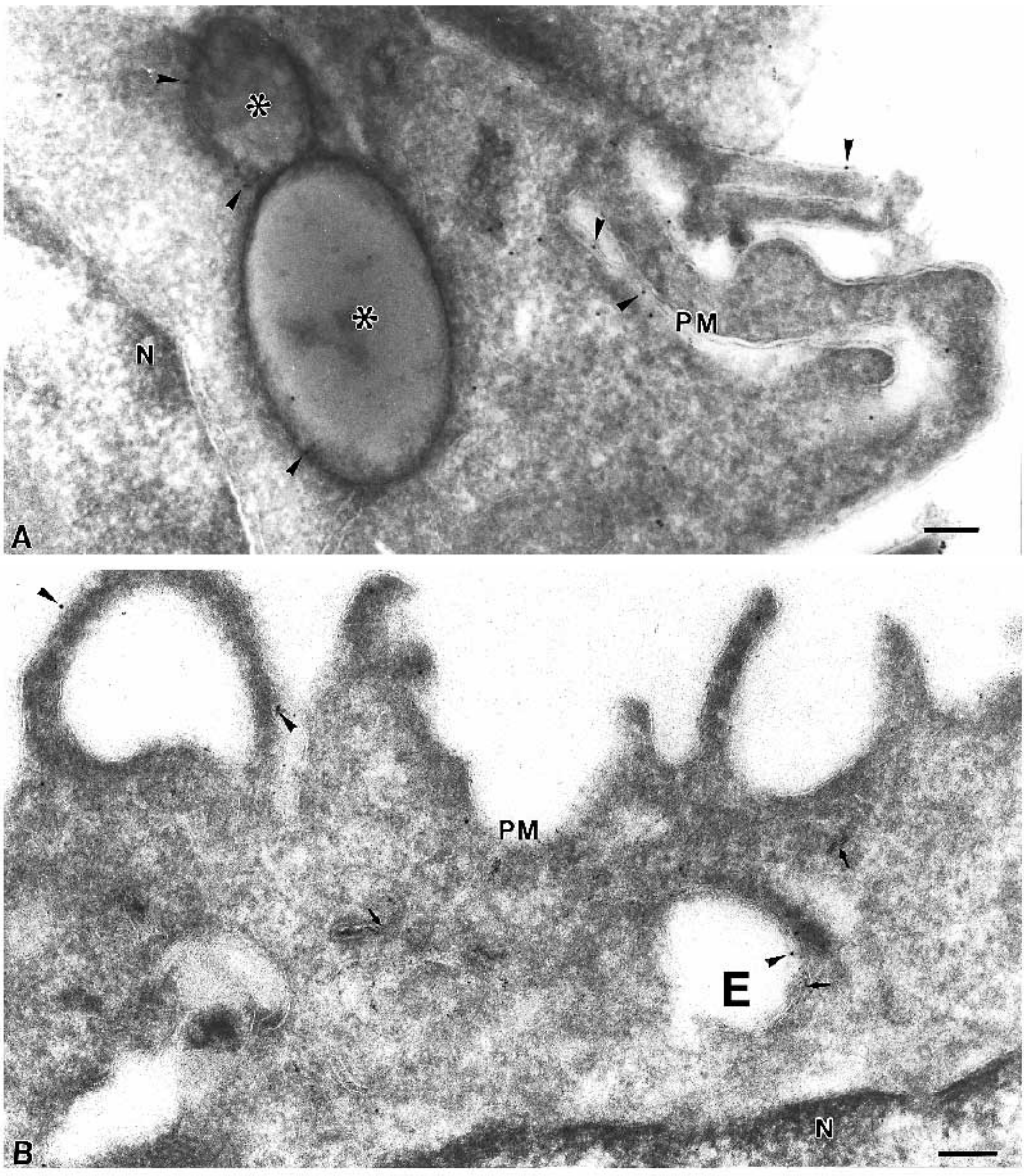

Fig. 7. Immunolocalization of annexin IV in J774 cells. Annexin IV labeling (small arrowheads) was seen on the plasma membrane (PM) and phagosomes (A, asterisks). In contrast, in B there is only one gold particle labeling the early endosomes (E) identified by the 5 nm gold internalized for 5 minutes (small arrows). N, nucleus. Bars, 100 nm.

associated with phagosomes is also considerable, ranging from approximately half the amount of the plasma membrane in the case of annexin I, IV and V to almost the same levels in the case of annexins II and III.

Phagocytosis of opsonized beads

Since latex beads are inert particles lacking specific ligands we asked where similar beads opsonized with IgG would behave differently with respect to the localization of annexins. For this we compared by immunofluorescence microscopy the localization of annexins I and II on opsonized and non-opsonized 5 μm beads that had been internalized into phagosomes of J774 cells. These larger beads make it easier at the immunofluorescence level to more clearly visualise phagosomal labeling. We could not detect any significant difference in the localization of either annexin I (not shown) or annexin II (Fig. 12A-D) between the two different kinds of phagosomes. For comparison, the labeling for Lamp2, which labels phagosomes strongly, is shown in Fig. 12E and F. We also performed a quantitative analysis on the labeling of annexins I-V on phagosomes containing 1 μm latex beads, that were either opsonized or not, by immunogold electron microscopy and cryosections. For all the annexins there was no significant quantitative difference in labeling of phagosomes containing opsonized beads relative to the values obtained with naked beads (results not shown).

DISCUSSION

The present study describes our qualitative and quantitative analyses of the subcellular distribution of five annexins at the electron microscope level. Our results show that whereas all five annexins are present on both the plasma membrane and phagosomes, the localization on other organelles differs. While annexins I, II, III and V were detected on early endosomes,

only annexin V was seen on late endocytic organelles and mitochondria.

Since all annexins studied were found on latex bead phagosomes we investigated this point in more detail since these organelles can be easely purified from J774 cells. To determinate whether phagosomal binding of annexins was transient, we obtained phagosomal fractions at different time points of phagosome maturation and analysed them by western blotting. In conjunction with our earlier data (Desjardins et al., 1994b) we conclude that four annexins (I, II, III and V) are present at similar levels on phagosomes of all ages until 24 hours, whereas the levels of annexins VI (Desjardins et al., 1994) and IV (this study) increase during phagosome maturation. In additional, annexin III was identified on phagosomal membrane in a form that required micromolar levels of Ca^{2+} for the association. The presence of annexins I, II, IV and V in phagosome fractions is also clearly Ca^{2+}-dependent, as it is affected by the EDTA treatment. However, in contrast to annexin III the other annexins appeared to be more stably bound to the phagosomes at relatively low Ca^{2+} concentrations. In the case of annexin II its association with the early endosomes has recently been shown to be Ca^{2+}-independent (Jost et al., 1997). Our data here therefore argue that annexin II is not bound to the phagosome membrane by the same mechanism.

Our results add to an accumulating set of data on the intracellular localizations of different annexins in cells. Annexin I has recently been localized on the plasma membrane in MDCK and BHK cells by immuno electronmicroscopy (Seemann et al., 1996), while annexin II was also found on plasma membrane of BHK cells (Emans et al., 1993; Harder et al., 1997) and colocalizes with the cortical actin network in regions underneath the apical and basolateral membranes of polarised

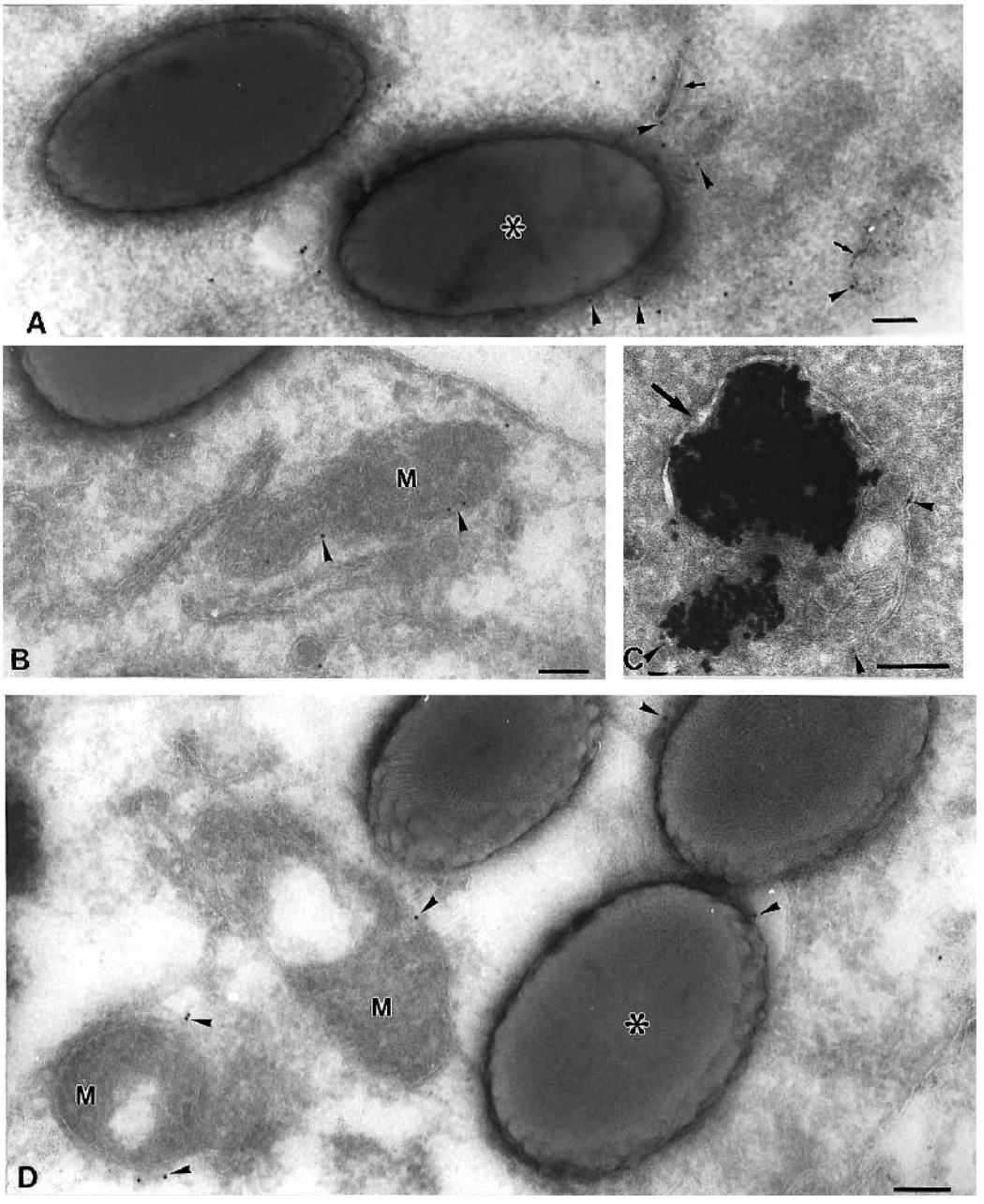

Fig. 8. Immunolocalization of annexin V in J774 cells. Annexin V labeling (small arrowheads) is shown on early endosomes (small arrows in A), phagosomes (A and D, asterisks) and on the membrane of late endocytic elements (C, large arrows). Mitochondria (M in B and D) also showed significant labeling with this antibody. Bars, 100 nm.

monolayers of MDCK cells (Harder and Gerke, 1993). Annexin V was found partly associated with cytoskeletal structures and partly with the plasma membrane of chicken-embryo fibroblasts (Koster et al., 1993). The presence of annexin V was also demonstrated on microvilli of the syncytiotrophoblasts (Krikun et al., 1994) and adjacent to the sacrolemma in the cardiac myocyte (Jans et al., 1995). In the present study we found not only annexins I, II and V but also III and IV on the plasma membrane of J774 macrophages and, additionally, showed that the distribution of annexins I and II along the plasma membrane is non-uniform; in both cases there was a significant concentration of antigen in areas of cell ruffles where it co-localized with F-actin.

There are a number of reports describing the localization of different annexins along the endocytic pathway (see Introduction). Annexins I and II were previously detected on early endosomes of both BHK (Emans et al., 1993; Seemann et al., 1996) and MDCK cells (Harder and Gerke, 1993; Seemann et al., 1996). In this study we detected significant amounts of annexins I, II, III and V, but not IV, on early endosomes of J774 macrophages. Annexin I (Futter et al., 1993) and annexin VI (Jackle et al., 1994) have previously been detected on multi-

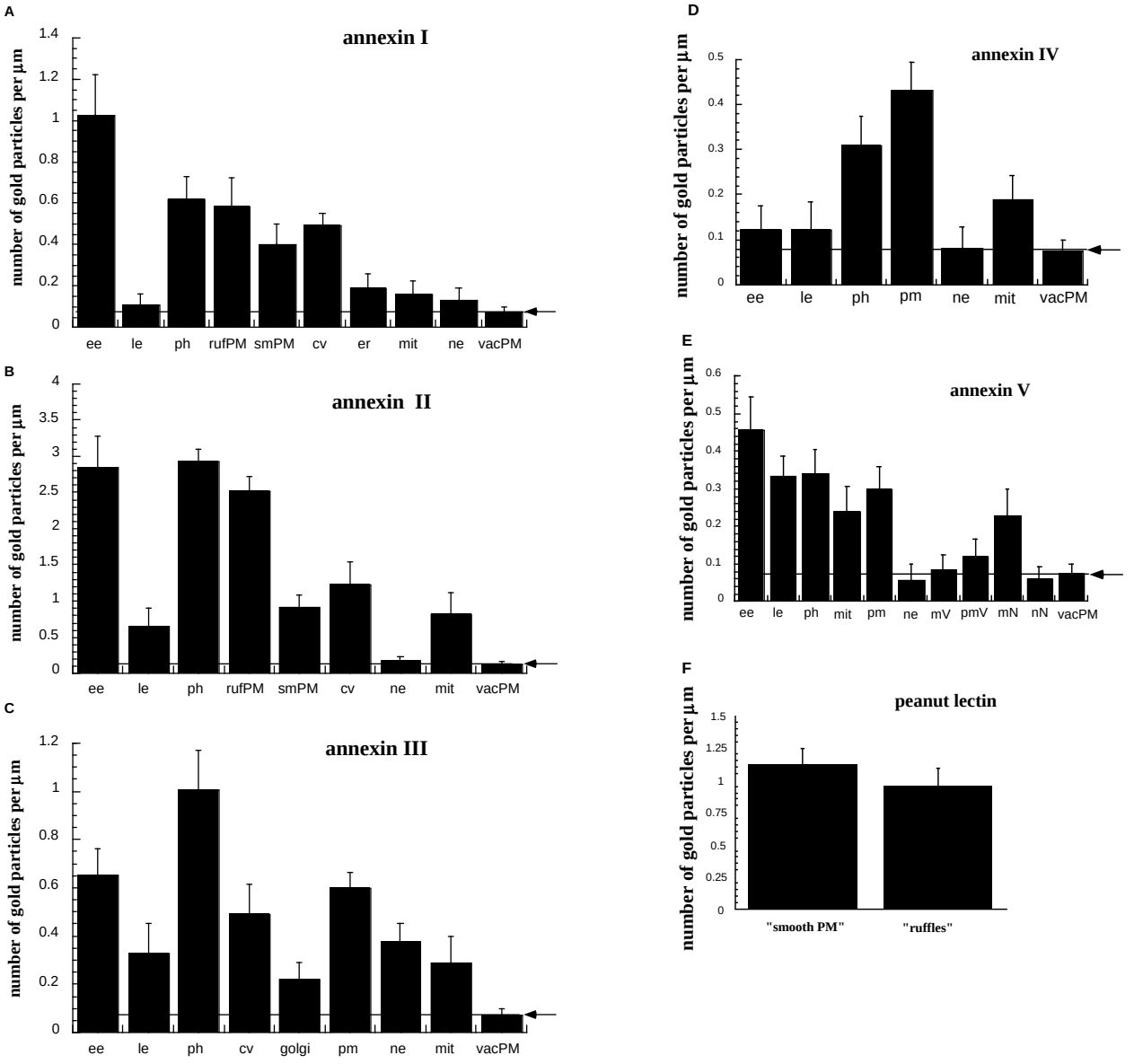

Fig. 9. Quantification of the immunogold labeling of annexins I-V in J774 cells. The density of gold labeling per linear µm of organelle membrane profile is given for the five annexins. Abbreviations used are: ee, early endosomes; le, late endocytic organelles; ph, phagosomes; mit, mitochondria; pm, plasma membrane; smPM, 'smooth' part of the plasma membrane; rufPM, ruffles and microvilli; er, endoplasmic reticulum; cv, coated vesicles; ne, nuclear envelope; golgi, Golgi complex; mV, mitochondria labeled with irrelevant anti-mouse hepatitis virus M protein antibody; pmV, plasma membrane labeled with anti-mouse hepatitis virus M protein; mN, mitochondria of NRK cells; nN, nuclear envelope of NRK cells. Arrows indicate the average level of labeling obtained with irrelevant anti-vaccinia virus antibodies (vacPM) (background). F shows a relative quantitation of gold labeling the peanut lectin over ruffles-microvilli versus the 'smooth' part of the plasma membrane.

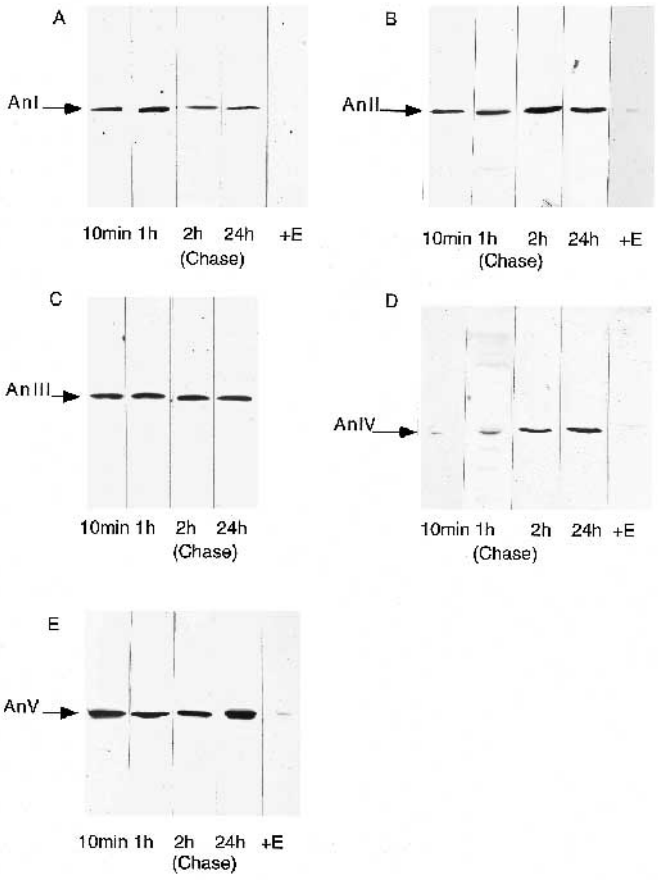

Fig. 10. Western blotting of phagosome fractions with anti-annexin antibodies. Phagosomes were formed by internalization of latex beads for either 20 minutes followed by a 10 minute chase (each left lane) or 60 minutes followed by further incubation without beads for 1, 2 or 24 hours. In some experiments all solutions were supplemented with 0.5 mM EDTA for the latest time point (1 hour pulse and 24 hours chase) (+E). Note that the presence of annexins I, II, IV and V in the phagosome fractions was affected by the addition of EDTA. Annexin III was detected in phagosome fractions only when all solutions were supplemented with 100 μM Ca^{2+}. Nitrocellulose filters were incubated with anti-annexin I (A), anti-annexin II (B), anti-annexin III (C), anti-annexin IV (D) and anti-annexin V (E) antibodies.

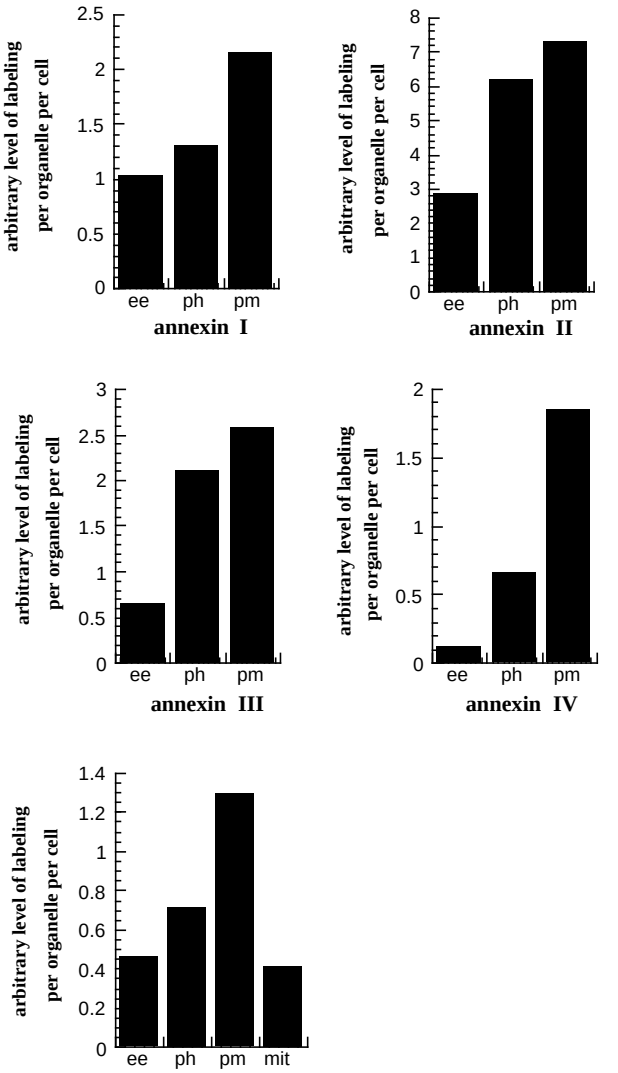

Fig. 11. Concentration of gold particles normalized to the relative surface areas of the membranes of early endosomes, phagosomes, plasma membrane and mitochondria (for annexin V only). These values are obtained by multiplying the concentration of gold per μm of boundary length of membrane by the relative surface area obtained by intersections counts. For example, there was, on average, 1 gold per μm for annexin I on the early endosomes (see annexin I) and 0.5 gold per μm on the plasma membrane (the average of 'smooth' and 'ruffled' parts). Since, however there is 4.3 times more membrane on the plasma membrane than on early endosomes per cell the arbitrary total amount of antigen is $4.3 \times 0.5 = 2.15$ arbitrary gold units per cell for the plasma membrane and $1 \times 1 = 1$ unit for early endosomes.

vesicular bodies that are located downstream of the early endosomes in the endocytic pathway. These structures have not yet been seen in J774 macrophages. In contrast, on the late endocytic compartments labeled by an overnight chase with gold particles we could not detect significant labeling for any of the annexins, except annexin V.

It is tempting to speculate that the significance of finding all five annexins on the plasma membrane and phagosomes is related to the fact that both these membranes are enriched in actin. Indeed, probably the bulk of cellular F-actin is directly or indirectly attached to the plasma membrane. Moreover, interactions between annexins and F-actin have been reported in vitro and in situ. Annexin I, for example, binds to (Schlaepfer and Haigler, 1987) and bundles (Glenney et al., 1987) F-actin in vitro, and this annexin has been shown by immonofluorescence microscopy to colocalize with the cortical actin in human fibroblasts (Glenney et al., 1987) and with filamentous actin at the sites of membrane ruffles of T51 rat liver cells after EGF stimulation (Campos-Gonzales et al., 1990). Recently, annexin I has also been found to interact with the actin-binding protein profilin (Alvarez-Martinez et al., 1996). Annexin II, complexed with its cellular protein ligand p11 in an annexin II_2p11_2 heterotetramer, is also associated with submembranous cytoskeleton and the cytoplasmic face of the plasma membrane (Greenberg and Edelman, 1983; Zokas and Glenney, 1987; Glenney et al., 1987; Osborn et al., 1988; Semich et al., 1989). This complex can bind to, and bundle F-

actin in vitro (Gerke and Weber, 1984; Glenney et al., 1987; Ikebuchi and Waisman, 1990). More recently, annexin II has been found as a part of a complex containing membrane-associated actin, α-actinin, ezrin and moesin (Harder et al., 1997). Non-erythroid spectrin, another actin-binding component of the submembranous cytoskeleton can also bind to the annexin II complex in vitro (Gerke and Weber, 1984). For these reasons it has been proposed that annexins (at least, annexins I and II) can serve a structural role by linking membranes to the actin cytoskeleton (Glenney, 1987; Gerke, 1989; Harder et al., 1997).

The second postulated role of annexins relevant to our study is in membrane docking/fusion. All annexins bind phospholipids in the presence of Ca^{2+}. Annexin I has been shown to mediate Ca^{2+}-dependent fusion of phosphatidylserine liposomes with themselves (Blackwood and Ernst, 1990; Meers et al., 1992), as well as the fusion of liposomes with the neutrophil plasma membrane (Oshry et al., 1991). Moreover, annexins I, II, IV and VII have been shown to bind to and promote the Ca^{2+}-dependent aggregation of secretory granules in vitro, suggesting that these proteins could play a role during exocytosis (Creutz et al., 1978, 1987; Drust and Creutz, 1988; Burgoyne, 1988; Blackwood and Ernst, 1990; Meers et al., 1992; for review see Creutz, 1992; Burgoyne and Morgan, 1993; Raynal and Pollard, 1994). However, while the annexin II tetramer is able to promote chromaffin granule aggregation

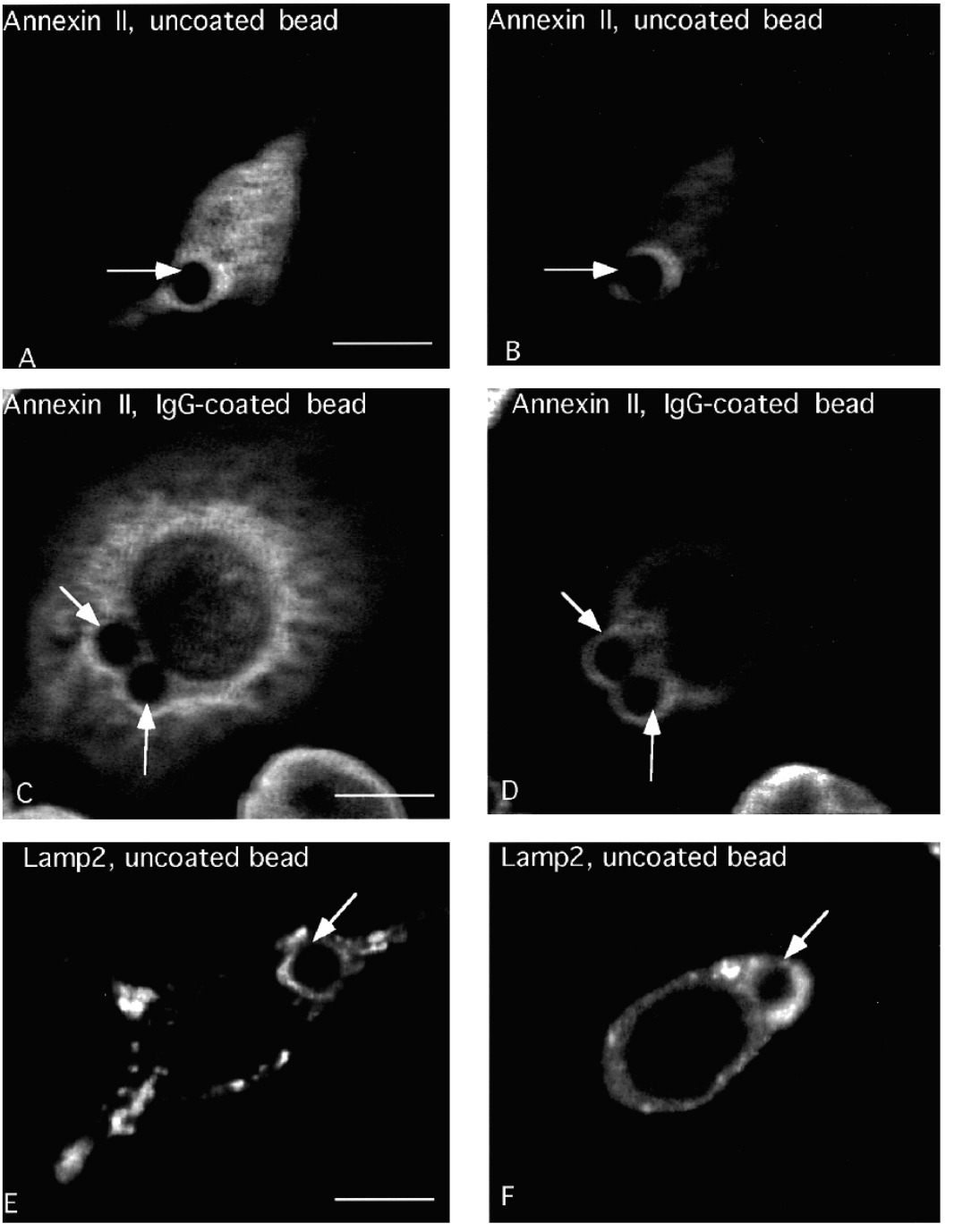

Fig. 12. Distribution of annexin II in J774 cells during phagocytosis of uncoated and opsonized latex beads visualized by confocal microscopy. Phagosomes (arrows) were formed by internalization of either 5 μm uncoated latex beads (A,B,E,F) or 5 μm beads conjugated with IgG (C,D). After methanol fixation cells were labeled by anti-annexin monoclonal antibody (A-D). Anti-lamp2 labeling was performed as a positive control for marking phagosomes with 5 μm beads (E,F). To demonstrate more clearly the higher fluorescence pattern around phagosomes we show two optical sections at different levels. A-B, C-D and E-F are paired optical sections. Bar, 10 μm.

at low Ca^{2+} concentration (about 1 μM) and to fuse them after addition of arachidonic acid (Drust and Creutz, 1988), other annexins need significantly higher (non-physiological) concentrations of Ca^{2+} to promote membrane-membrane contact (Zaks and Creutz, 1991). A recent study has shown that annexin VII (synexin) can facilitate aggregation and fusion of chromaffin granules in the presence of 50-200 μm calcium by a novel GTP-dependent mechanism (Caohuy et al., 1996).

Using permeabilized chromaffin cells exogenously added annexin II has been shown to stimulate Ca^{2+}-induced catecholamine secretion, suggesting a role for this protein in Ca^{2+}-regulated exocytosis (Ali et al., 1989; Sarafian et al., 1991; Creutz, 1992). Moreover, ultrastructural analyses indicated that the annexin II tetramer changes its conformation and cross-links secretory vesicles with the plasma membrane after stimulation of chromaffin cells (Nakate et al., 1990). It was recently shown that annexin II tends to accumulate in the subplasmalemma of chromaffin cells, concomitant with catecholamine secretion (Chasserot-Golaz et al., 1996). This led to the suggestion that the role of this annexin is not to direct the granules towards the exocytotic sites but to promote membrane fusion itself. Moreover, it has been speculated that annexin II participates in membrane fusion at all stages of the hepatocyte transcytotic pathway (Wilton et al., 1994).

Annexin II has also been implicated in a docking and/or fusion process between early endosome in vitro (Emans et al., 1993; Mayorga et al., 1994) and in vivo (Emans et al., 1993; Harder and Gerke, 1993). During the in vitro fusion between early endosomes from BHK cells annexin II is selectively transferred from the membranes of the donor endosomes to the acceptor endosomes (Emans et al., 1993). Furthermore, anti-annexin II antibodies, as well as an annexin II peptide blocked calcium-dependent fusion by 50% in a cell-free endosome fusion assay (Mayorga et al., 1994). In all these studies it remains unclear whether the annexins facilitate membrane fusion itself or, as proposed by Creutz (1992) they play a more upstream role in the docking step.

While our results do not allow us to add to the present discussion on the role of annexins in membrane trafficking, our finding describing the localization of five annexins on phagosomes may be related to the interaction of these specialised organelles with either actin, other endocytic compartments, or both. It is well established that the actin cytoskeleton is essential for the formation of phagosomes. While a number of reports have suggested that, after their formation the phagosome-bound actin is lost (Silverstein et al., 1989; Greenberg et al., 1991) actin can be detected associated with isolated latex bead phagosomes at all times after their formation (up to 48 hours) (Desjardins et al., 1994a, and unpublished data). Our latex bead-containing phagosomes can also specifically bind actin in a novel in vitro binding assay (Shonn et al., 1995) but it remains to be seen whether any annexins are involved in this process. Latex bead phagosomes can also fuse with elements of the endocytic pathway and they do so in an age-dependent fashion. Whereas phagosomes up to 12 hours of age fuse avidly in vitro with enriched populations of early endosomes, late endosomes as well as lysosomes, they essentially lose this fusion capability at later times (A. Jahraus et al., unpublished), a finding supported by in vivo observations (M. Desjardins et al., unpublished). It is tempting to speculate that this loss of fusion capability may be related to the increase we observe in the amount of phagosome-associated annexins IV and VI at these later time points.

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