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Plasticity of early endosomes

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Summary

We observed that the structural organization of early endosomes was significantly modified after cell surface biotinylation followed by incubation in the presence of low concentrations of avidin. Under these conditions early endosomes increased in size to form structures which extended over several micrometres and which had an intra-luminal content with a characteristic electron-dense appearance. The modified early endosomes were not formed when either avidin or biotinylation was omitted, suggesting that they resulted from the cross-linking of internalized biotinylated proteins by avidin. Accumulation of a fluid-phase tracer was increased after the avidin-biotin treatment (145% after 45 min). Both recycling and transport to the late endosomes still occurred, albeit to a somewhat lower extent than in control cells. Quantitative electron microscopy showed that the volume of the endosomal compartment was increased approximately 1.5-fold but that the surface area of the compartment decreased relative to its volume after avidin-biotin treatment. Finally, overexpression of a rab5 mutant, which is known to inhibit early endosome fusion in vitro, prevented the formation of these structures in vivo and caused early endosome fragmentation. Altogether, our data suggest that early endosomes exhibit a high plasticity in vivo. Cross-linking appears to interfere with this dynamic process but does not arrest membrane traffic to/from early endosomes.

Key words: electron microscopy, internalization, avidin-biotin, small GTP-binding protein rab5, membrane fusion.

Introduction

It is now clear that internalized tracers first appear in early endosomes at the cell periphery and then in late endosomes in the perinuclear region, before they are observed in lysosomes (Gruenberg and Howell, 1989; Hubbard, 1989; Kornfeld and Mellman, 1989). However, relatively little is known about the structural and functional organization of early and late endosomes. Morphologically, early endosomal elements are highly polymorphic, consisting of cisternal, tubular and vesicular regions or elements (Wall et al., 1980; Geuze et al., 1983; Hopkins, 1983; Gruenberg et al., 1989; Griffiths et al., 1989). Tubular regions were proposed to contain receptors destined to be recycled, and vesicular regions ligands destined to be degraded (Geuze et al., 1983). In fact an extensive tubular endosomal network was observed in Hep2 cells (Hopkins et al., 1990). More recently, electron microscopy studies have shown that presumably the same network is part of the early endosome and is organized in separate unconnected clusters (Tooze and Hollinshead, 1991). Interestingly, the extent of this network varied between cell lines, being extensive in Hep2 cells but not in the BHK cells which we used in the present study.

In cell-free assays, early endosomes exhibit a striking tendency to undergo fusion with each other (Davey et al., 1985; Gruenberg and Howell, 1986,1987; Braell, 1987; Diaz et al., 1988; Woodman and Warren, 1988; Gruenberg et al., 1989). This process is specific (Gruenberg et al., 1989; Bomsel et al., 1990), microtubule-independent (Gruenberg et al., 1989; Bomsel et al., 1990), inhibited by the cell cycle control protein kinase cdc2 (Tuomikoski et al., 1989), regulated by NSF (Diaz et al., 1989) and by the small GTP-binding protein rab5 (Gorvel et al., 1991). We have previously proposed that this high fusion activity in vitro suggests that in vivo early endosomes form a dynamic network of individual elements interacting via fission and fusion events (Gruenberg et al., 1989). It has been difficult to follow these events in vivo, because of the rapid kinetics of transport in the endocytic pathway. Internalized markers leave the early endosome within minutes after internalization (Storrie et al., 1984; Dunn and Hubbard, 1984; Mueller and Hubbard, 1986; Tran et al., 1987; Goldenthal et al., 1988; Gruenberg et al., 1989; McVey Ward et al., 1990). However, two sequentially internalized tracers can meet in early endosomes, provided that the two pulses are relatively close (Salzman and Maxfield, 1988; McVey Ward et al., 1990).

In the present study, we have made use of a cell surface biotinylation protocol (Lisanti et al., 1988; Le Bivic et al., 1989), followed by avidin internalization, to investigate the plasticity of early endosomes in vivo. Our data suggest that the structural appearance of early endosomes can be significantly changed, presumably via cross-linking of the

luminal membranes of early endosomes, without causing major changes in the pathways of membrane traffic to/from early endosomes.

Materials and methods

Cells

Monolayers of baby hamster kidney (BHK) cells were maintained as previously described and plated 14 h before use (Gruenberg et al., 1989). To label late endosomes and lysosomes, the cells were incubated for 3 h with 16 nm BSA-gold 2 h after passage and the gold was then chased overnight (Griffiths et al., 1988). To label early compartments cells were incubated with 10 mg/ml horseradish peroxidase (HRP grade II, Sigma Chemical Co., or from Serva GMBH, Heidelberg) for up to 30 min at 37°C. As in a previous study (Gorvel et al., 1991), biotinylation of the cell surface was carried out with the protocol of Le Bivic et al. (1989), using a cleavable derivative of biotin (biotin-X-SS-NHS, sulphosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate; Pierce). When indicated, the biotin residues present on the cell surface were cleaved with the non-permeant reagent glutathione (Bretscher and Lutter, 1988; Graeve et al., 1989). Detection of the cell surface biotin residues was carried out by fluorescence microscopy using FITC-streptavidin. Experimental manipulation of the cells was carried out on ice unless stated otherwise.

Infection using the recombinant rab5 vaccinia virus was performed exactly as described (Bucci et al., 1992). Over 90% of the cells overexpressed the rab5 or rab5Ile133 proteins. Overexpression was approximately 5-fold over uninfected cells.

Formation of modified endosomes

The typical conditions we used to modify the structural organization of early endosomes were to co-internalize cell surface biotinylated proteins with avidin present in the medium at low concentrations. After cell surface biotinylation, the monolayers were incubated at 37°C for 60 min in internalization medium (IM: MEM, 10 mM Hepes, pH 7.4, 10 mM D-glucose, 0.2% BSA), with or without 0.1 mg/ml avidin. The cells were then returned to ice temperature, washed for 3 × 10 min with PBS containing 5 mg/ml BSA (PBS-BSA) and either processed for electron microscopy (or fluorescence microscopy) or further manipulated as described below.

Quantification of internalization, recycling and transport to late endosomes

To quantify fluid-phase endocytosis, the cells were incubated at 37°C in IM containing 2 mg/ml HRP for different times, as indicated in the text. The cells were then returned to ice temperature, washed 3× in PBS-BSA as above and the amount of cell-associated HRP was quantified (Gruenberg et al., 1989). Recycling from the early endosomes was measured after loading the early endosomes with HRP for 5 min at 37°C. The cells were then washed 3× in PBS-BSA, as above, reincubated in HRP-free medium for different times at 37°C, and the amount of cell-associated HRP was quantified.

Transport to late endosomes was measured after subcellular fractionation. The early endosomes were first labeled with HRP, as above, and then HRP was chased into the late stages of the endocytic pathway by re-incubating the cells for 45 min at 37°C in HRP-free IM. The cells were then fractionated using a flotation step gradient that separates early and late endosomes (Gorvel et al., 1991; Chavrier et al., 1991). The post-nuclear supernatant was brought to 40.6% sucrose and overlaid sequentially with 35% sucrose, 3 mM imidazole, pH 7.4, and 25% sucrose, 3 mM imidazole, pH 7.4, and finally with homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4). The gradient was run for 60 min at 35,000 revs/min using an SW-60 rotor. The early endosomes were recovered at the 35%/25% interface and the late endosomes in the uppermost portion of the 25% sucrose. The amount of HRP in the different subcellular fractions was then quantified. Protein determination was carried out with the protocol of Bradford (1976).

Electron microscopy

For Epon embedding, cells were fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer. Semi-thick (250-300 nm) sections of Epon-embedded material were usually cut parallel to the substratum. However, for quantitative studies sections were cut perpendicular to the substratum to generate random vertical sections (Baddeley et al., 1986). Quantitation of the volume of the HRP-labelled compartment relative to the cytoplasmic volume and of the surface area to volume ratios of the labelled structures was by intersection and point-counting as previously described (Parton et al., 1989). The reference space for the quantitation (cytoplasmic volume) was not changed by the avidin-biotin treatment.

Cells to be processed for frozen sectioning were fixed in 8% paraformaldehyde, 250 mM HEPES, pH 7.4, and then were scraped from the culture dish and pelleted. Frozen sectioning, preparation of streptavidin-gold and labelling was as previously described (Griffiths et al., 1983; Brändli et al., 1990).

Results

Internalization of biotinylated proteins

We used a biotin derivative containing a disulphide bridge to biotinylate the plasma membrane of BHK cells (Lisanti et al., 1988; Le Bivic et al., 1989). Fluorescence microscopy using FITC-streptavidin (Fig. 1A) and electron microscopy using streptavidin-gold (see Fig. 2A) showed that the labelling was restricted to the cell surface. Reduction of the disulphide bridge with glutathione, a non-permeant reducing agent, abolished labelling (Figs 1B and 2B). Upon incubation at 37°C for 60 min, a fraction of the biotinylated proteins was internalized and distributed predominantly in peripheral endosomal structures, which are visualized in Fig. 1C using FITC-streptavidin. Labelling of cryo-sections with streptavidin-gold showed that labelled structures exhibited the characteristic cisternal and tubulo-vesicular morphology of early endosomes (Fig. 2C,D). That internalized biotinylated proteins are present predominantly in early endosomes and not in late endosomes is consistent with the observation that most internalized plasma membrane proteins rapidly recycle back to the cell surface (Haylett and Thilo, 1986; Draye et al., 1987). However, it is also possible that a fraction of the biotinylated proteins was transported to late endocytic compartments, where the label was lost via degradation, or reduction of the disuphide bridge of biotin.

Endosomes after avidin internalization

When avidin at relatively low concentrations was co-internalized for 60 min at 37°C after cell surface biotinylation, the organization of endosomes became strikingly different. In thawed cryo-sections of fixed cells, large structures were then observed that could be labelled with antibodies against avidin followed by Protein A-gold (Fig. 3). Since these structures could also be labelled with streptavidin-gold,

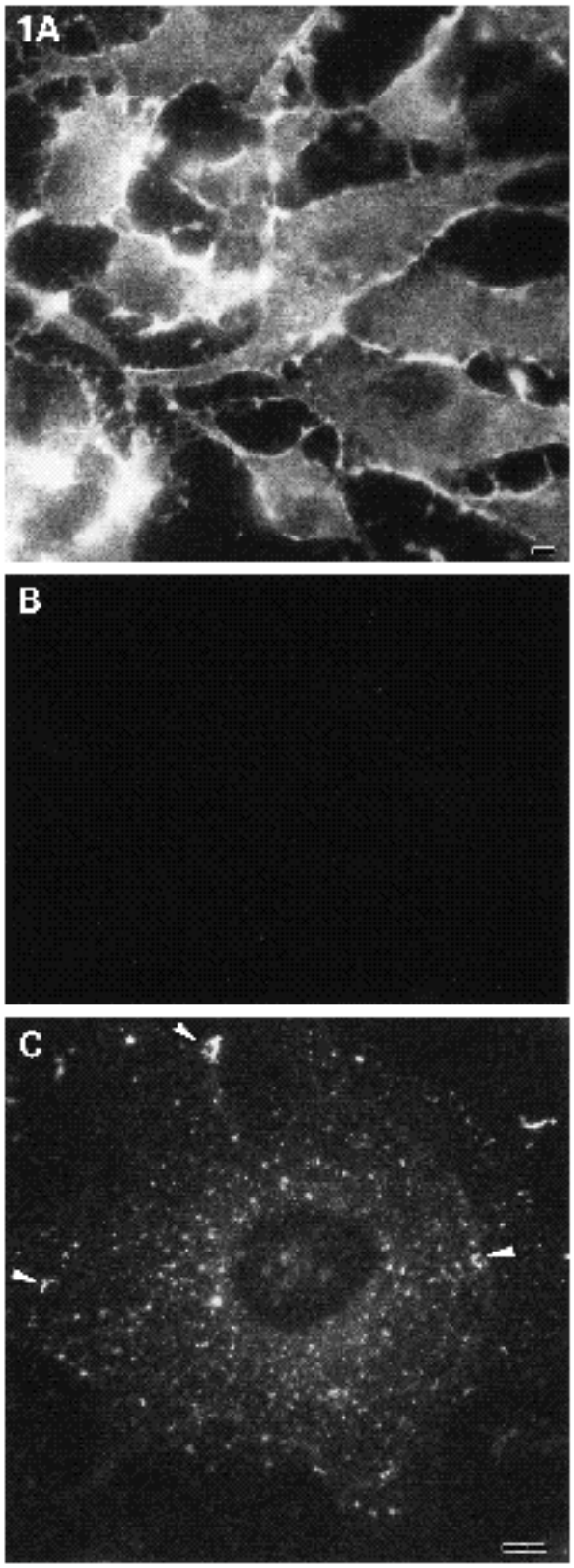


Fig. 1. Detection of biotinylated proteins by fluorescence. The cell surface was biotinylated using a biotin reagent containing a disulphide bridge. (A) The biotinylated proteins were visualized using FITC-streptavidin. (B) After reduction of the disulphide bridge with glutathione, no fluorescence could be detected. (C) After biotinylation, the cells were incubated for 60 min at 37°C, returned to ice temperature and the biotin residues still present on the cell surface were cleaved with glutathione. The cells were then fixed, permeabilized with Triton X-100 and the internalized biotinylated proteins were labelled with FITC-streptavidin. Labelling is evident within small structures distributed throughout the cell. Arrowheads indicate larger endosomal structures. Bars, 1 μm (A and B same magnification).

some free biotin groups must have remained available in endosomes after avidin addition. In the example shown in Fig. 3A, a complex and continuous network, labelled with both markers, extends over several micrometres. Similar structures were never observed in untreated BHK cells. In addition to changes in the average size of endosomal elements, the content of endosomes also appeared significantly altered. Regions of endosomes appeared more vacuolated and electron-dense, containing a complex organization of internal membranes that exhibited some resemblance to multivesicular endosomes (see for example, Dunn et al., 1986) and putative endosome carrier vesicles (Gruenberg et al., 1989; Bomsel et al., 1990) (Figs 3, 5). These vesicular regions were often connected together by tubular or cisternal elements. In some cases these regions were observed in continuity with curved cisternal elements surrounding an electron-lucent region (Fig. 3C). We will refer to these structures as modified endosomes.

The formation of modified endosomes was not due to biotinylation itself, since endosomal elements exhibited their typical cisternal and tubulo-vesicular appearance after internalization of biotin in the absence of avidin (Figs 2C and 4). Neither were these structures observed when the experiments were repeated in the presence of avidin without cell-surface biotinylation (not shown; see Table 1). Endosomes then appeared identical to those observed in control cells, indicating that avidin itself did not alter endosomal morphology. Moreover, modified endosomes did not form to any significant extent when biotinylated cells were incubated with a large excess of avidin (3 mg/ml instead of 0.1 mg/ml). Therefore we reasoned that these changes

Table 1. *Volume and surface area to volume ratio of endocytic organelles in biotin-avidin-treated cells*

Conditions	Volume density (% of cytoplasmic volume occupied by HRP-labelled structures ± s.e.m.)	Surface to volume ratio (± s.e.m.)
−Biotin +avidin, HRP, 30 min, 37°C	1.8 ± 0.4	1.6 ± 0.4
+Biotin, +avidin, HRP, 30 min, 37°C	2.6 ± 0.6	0.9 ± 0.2

Cells were treated with or without biotinylating reagents before incubation with 0.1 mg/ml avidin as described in Materials and methods; 10 mg/ml HRP was then internalized for 30 min before fixation and processing for Epon embedding. Quantification was performed on 20-30 random micrographs of thin sections cut perpendicular to the substratum (vertical sections) as previously described (Parton et al., 1989).

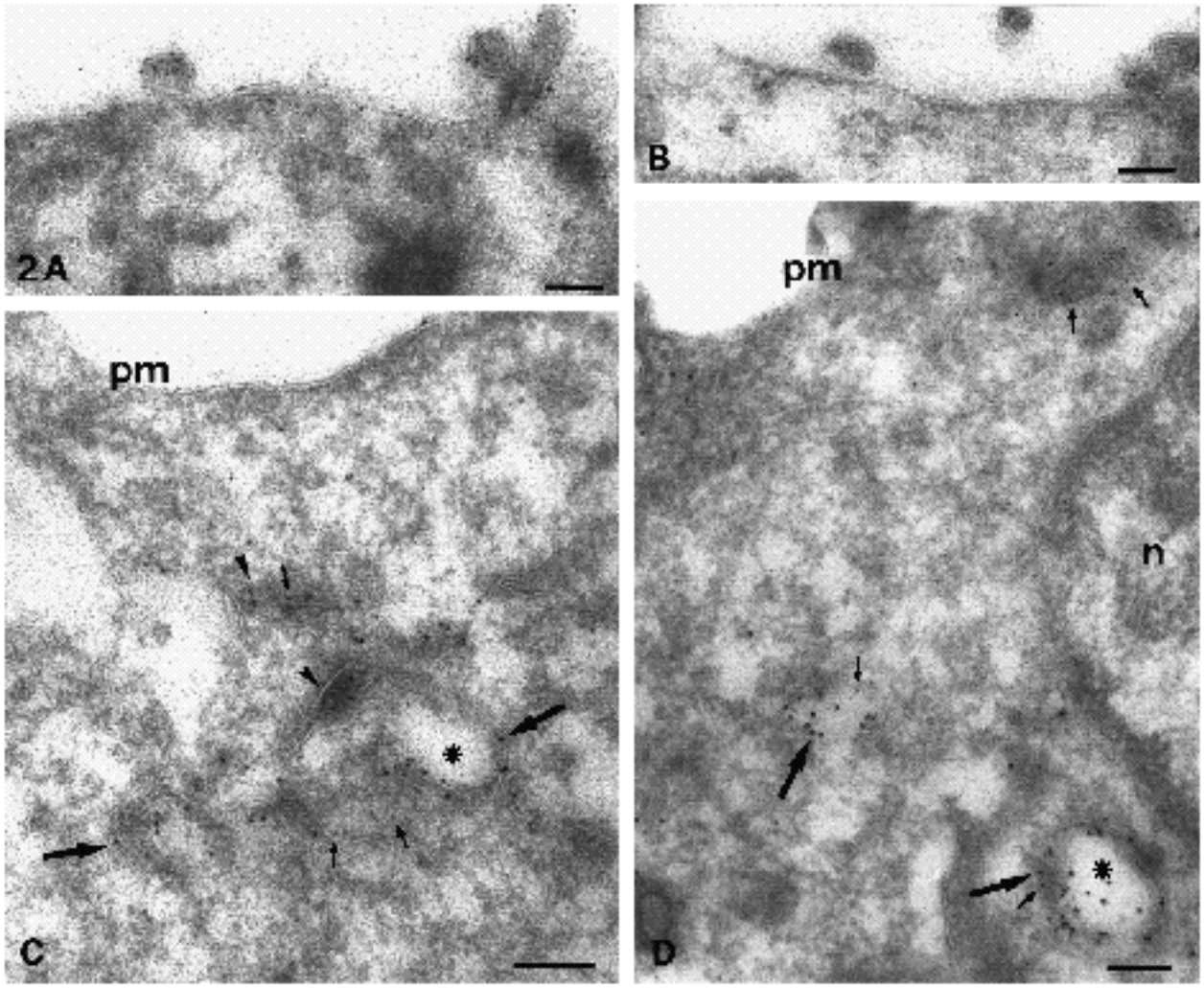


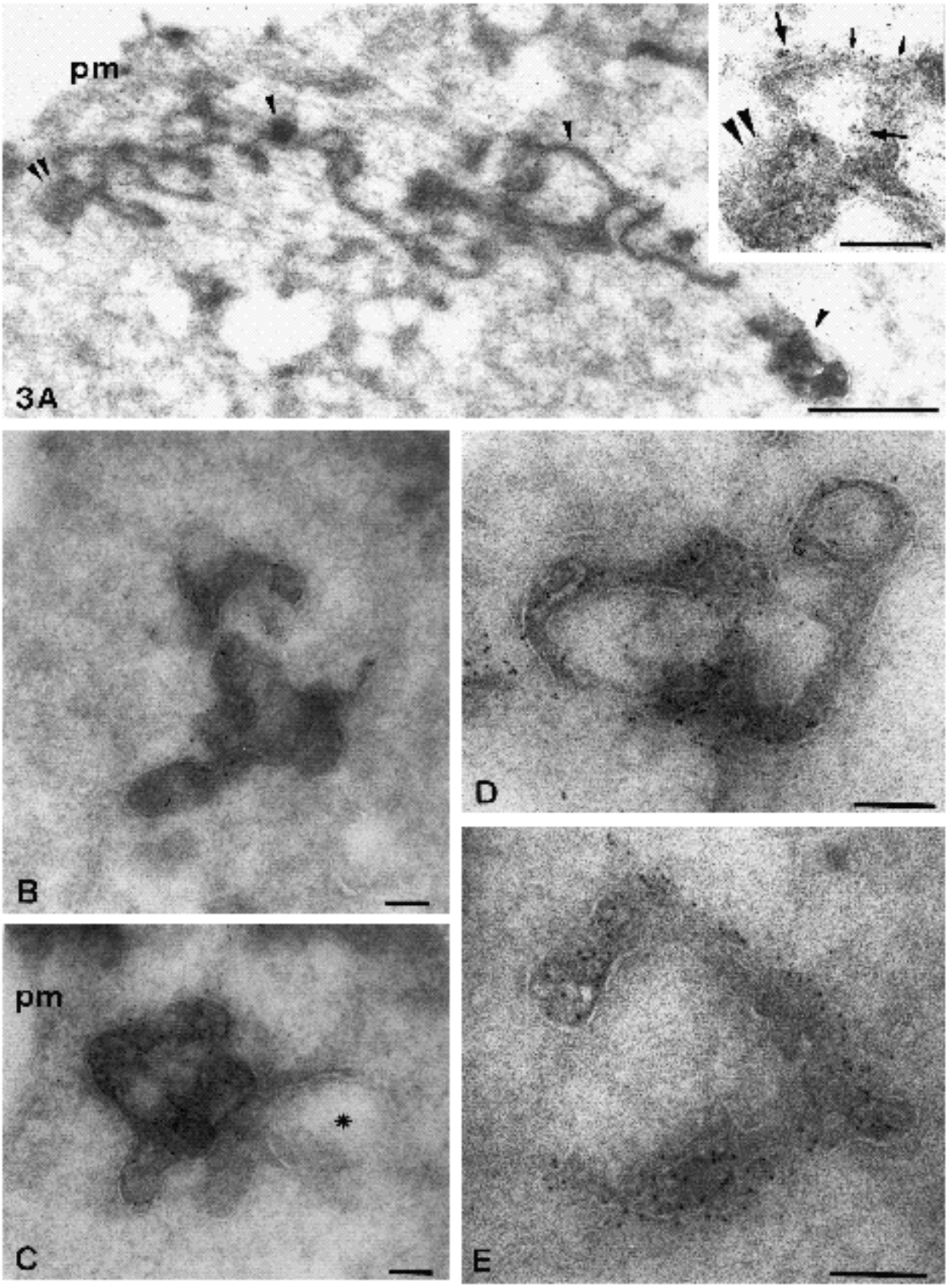
Fig. 2. Detection of biotinylated proteins on frozen sections using streptavidin-gold. After cell surface biotinylation cells were kept at 4°C (A,B) or incubated at 37°C for 60 min with HRP in the medium for the last 10 min (C,D). In (B,Cand D) the cells were then treated with glutathione to remove surface biotin groups. After fixation and preparation of frozen sections, labelled proteins were detected using streptavidin-gold (9 nm). In (A) the cell surface shows heavy labelling. This is reduced dramatically after glutathione treatment (B). (C,D) After internalization, streptavidin-gold labelling (9 nm gold, small arrows) is evident within early endosomal structures and colocalises with labelling for HRP (13 nm gold, large arrows). Labelling is evident within tubular profiles which often surround an electron-lucent area (asterisks) and occasionally within vesicular structures with a multivesicular content (arrowheads in C). pm, plasma membrane; n, nucleus. Bars, 0.25 μm.

probably result from the luminal cross-linking of a fraction of the internalized biotinylated proteins by avidin.

Avidin-biotin-modified endosomes are part of the early endosome

To characterize the modified endosomes further we examined their morphology in Epon sections. Before biotinylation, BSA-gold was internalized to label late endocytic compartments (late endosomes and lysosomes). After biotinylation of the cell surface and incubations with or without avidin, cells were incubated with HRP as a fluid-phase marker. After fixation and embedding in Epon, semi-

Fig. 3. Morphology of endosomal structures after co-internalization of biotinylated plasma membrane proteins and avidin. After biotinylation of the plasma membrane, the cells were incubated for 60 min at 37°C as in Fig. 2C and D but in the presence of 0.1 mg/ml avidin. (A) A low-magnification overview of the peripheral region of a cell in which an endosomal structure (indicated by arrowheads) extends over several micrometres. This structure is labelled with antibodies to avidin (6 nm Protein A-gold, small arrows) and with streptavidin-gold (9 nm gold, large arrows) as shown in the inset (higher magnification of the area indicated with double arrowheads). Similar structures are labelled with streptavidin-gold (B,C and D); or with anti-avidin antibodies followed by Protein A-gold (E). Note the electron density of the labelled structures and the complex organization of internal membranes. In C the abnormal multivesicular region of the labelled structure is clearly in continuity with a cisternal or tubular structure surrounding an electron-lucent space (asterisk) similar to those observed in control cells (see Fig. 2C,D). pm, plasma membrane. Bars: A, 1 μm (inset, 0.25 μm); B-E, 0.25 μm.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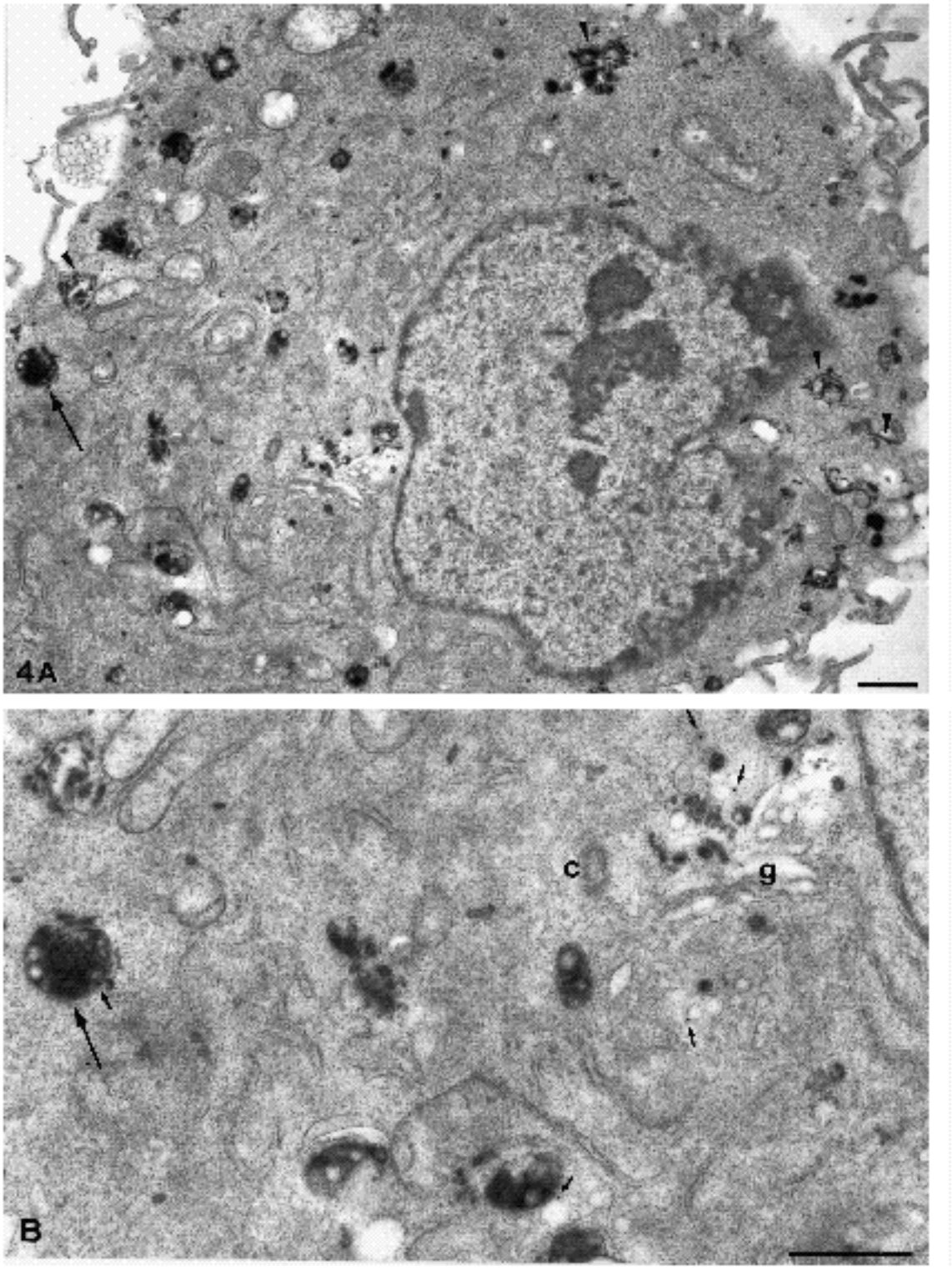

Figs 4-6 and 8 all show unstained semi-thick Epon sections (250 nm) which were cut parallel to the substratum.
Fig. 4. Semi-thick Epon sections of biotinylated (control) cells after internalization of HRP. Late endosomes and lysosomes were labelled with 16 nm BSA-gold which was internalized for 3 h at 37°C and then chased overnight. The cells were then surface-biotinylated and incubated at 37°C for 60 min (without avidin). During the last 30 min 10 mg/ml HRP was included in the medium. After fixation, HRP activity was visualized using DAB and cells were embedded in Epon. (A)HRP labels small peripheral clusters of early endosomal tubules (arrowheads) and vesicles. Late endosomes labelled with gold (small arrows) are located close to the Golgi (g), as shown at higher magnification in B. An HRP- and gold-labelled late endosome is indicated (large arrow) in A and B. c, centriole. Bars, 1 μm.

thick sections (approximately 250 nm thick) were prepared and examined unstained in order to visualize the extent of the endosomal compartments after the different treatments (see Tooze and Hollinshead, 1991). In control cells, which were biotinylated but not avidin-treated, a 30 min incubation with HRP-labelled vesicular and tubular endosomal structures dispersed in small groups around the periphery of the cell and close to the Golgi (Fig. 4). The pattern of HRP labelling was completely different in biotin-avidin-treated cells (Fig. 5). Large endosomal structures were the major labelled elements in these cells. These structures showed a complex three-dimensional structure with multivesicular regions connected to cisternal elements, and clearly corresponded to the modified endosomes observed in cryosections. Comparison with control cells (Fig. 4) shows that the formation of the modified endosomes was also accompanied by a decrease in the number of peripheral tubules and small vesicles (see below and Table 1). Within modified endosomal structures, little BSA-gold could be detected. Correspondingly, late endosomes and lysosomes, that were labelled with BSA-gold, only rarely contained detectable amounts of HRP even after a 30 min incubation at 37°C (Fig. 6), and their morphology was identical to that observed in control cells (Fig. 5, Fig. 6B). These observations suggest that the biotinylation/avidin treatment predominantly affected the organization of early endosomes and had little effect on late endosomes and lysosomes.

We then further investigated whether modified endosomes were part of the early or the late endosomes. To identify early endosomes, HRP was internalized for 10 min at 37°C in cells that had been pre-treated with biotin and avidin, as above. Previous studies on BHK cells showed that under these conditions early but not late endosomes are labelled (Griffiths et al., 1989). Late endosomes and lysosomes were labelled with BSA-gold, internalized for 3 h at 37°C and then chased overnight before the experiment. Fig. 6A shows that large endosomal structures, but not the gold-labelled late endosomes, could be labelled with a relatively short pulse of HRP.

Internalization and recycling

Since the biotin-avidin treatment significantly changed the structure of early endosomes, we tested whether transport through the early endosomes was modified. To provide a general marker of the endosomal content, we used HRP internalized in the fluid phase. First, we followed HRP internalization for different times at 37°C in cells that had been biotinylated and then incubated for 60 min in the presence of avidin. As is shown in Fig. 7A, similar amounts of HRP appeared to be internalized with or without the biotin-avidin treatment, but more HRP was accumulated in treated cells, corresponding to 145% of the control value after 45 min.

We then measured the extent of recycling from the early endosome after preloading the biotin/avidin-treated cells with HRP for 5 min at 37°C. The HRP-labelled cells were washed on ice and reincubated at 37°C for different times in HRP-free medium. Recycling of the endosomal content was similar after the treatment, although somewhat less efficient, when compared to control cells (Fig. 7B). After 15 min re-incubation, the treated cells contained approx. twice as much HRP as the control cells, corresponding to an increment ≈16% of the HRP preinternalized during 5 min. The finding that recycling was reduced after the treatment is consistent with our observation that more HRP was accumulated in the cells.

Quantitative EM was used to investigate whether the decreased recycling and increased accumulation of fluid was accompanied by an increase in the size of the endosomal compartment. A 30 min HRP incubation was used to ensure that the entire early endosomal compartment was labelled. The volume of the HRP-labelled compartment relative to the cytosolic volume after a 30 min HRP incubation in biotin-avidin-treated cells was compared with control cells (incubated with avidin but without prior biotinylation). As shown in Table 1, the volume density of the HRP-labelled compartment was increased 1.5-fold after the combined biotinylation and avidin treatment (2.6±0.6% of the cytoplasmic volume) as compared to control cells (1.8±0.4%). We also measured the surface of the labelled compartments (excluding any internal membranes which could be surface connected) relative to their volume. The surface area to volume ratio was decreased from 1.6±0.4 in control cells to 0.9±0.2 after avidin-biotin treatment. These observations strongly suggest that the treatment resulted in a loss of tubular elements and a shift towards larger endosomal elements.

Transport to late endosomes

Having established that the biotin-avidin treatment increased HRP accumulation and decreased its recycling, we then studied whether transport to late endosomes was also affected. First, we used morphology to follow the distribution of internalized HRP. Late endosomes and lysosomes were labelled as above with BSA-gold that had been chased overnight. After 10 min at 37°C, HRP was distributed within structures with the typical altered appearance of modified endosomes (Fig. 6A). However, when the cells were then washed and reincubated at 37°C, the reaction product of HRP clearly labelled late endosomes containing BSA-gold (Fig. 6, inset). Avidin itself, detected by immunogold labelling on cryosections, could also be transported to late endosomes and lysosomes, as expected (not shown). These experiments indicate that endocytosed markers could be transported to morphologically unmodified late endosomes after the biotin-avidin treatment.

It is more difficult to quantify the extent of passage from early to late endosomes. Our approach was to make use of a flotation gradient that separates rab5-positive early endosomes from mannose 6-phosphate receptor-positive and rab7-positive late endosomes (Gorvel et al., 1991; Chavrier et al., 1991). Early endosomes were labelled with HRP for 5 min at 37°C and the label was then chased into late endosomes by a subsequent incubation in HRP-free medium for 40 min at 37°C (Griffiths et al., 1988; Gruenberg et al., 1989). The cells were homogenized and a postnuclear supernatant was prepared and fractionated with the gradient. The early and late endosome fractions were collected and their HRP content analysed. Under these conditions, both early and late endosomes could be prepared with a yield of ≈ 25% over the homogenate and an enrichment factor of ≈ 15-fold (Gorvel et al., 1991).

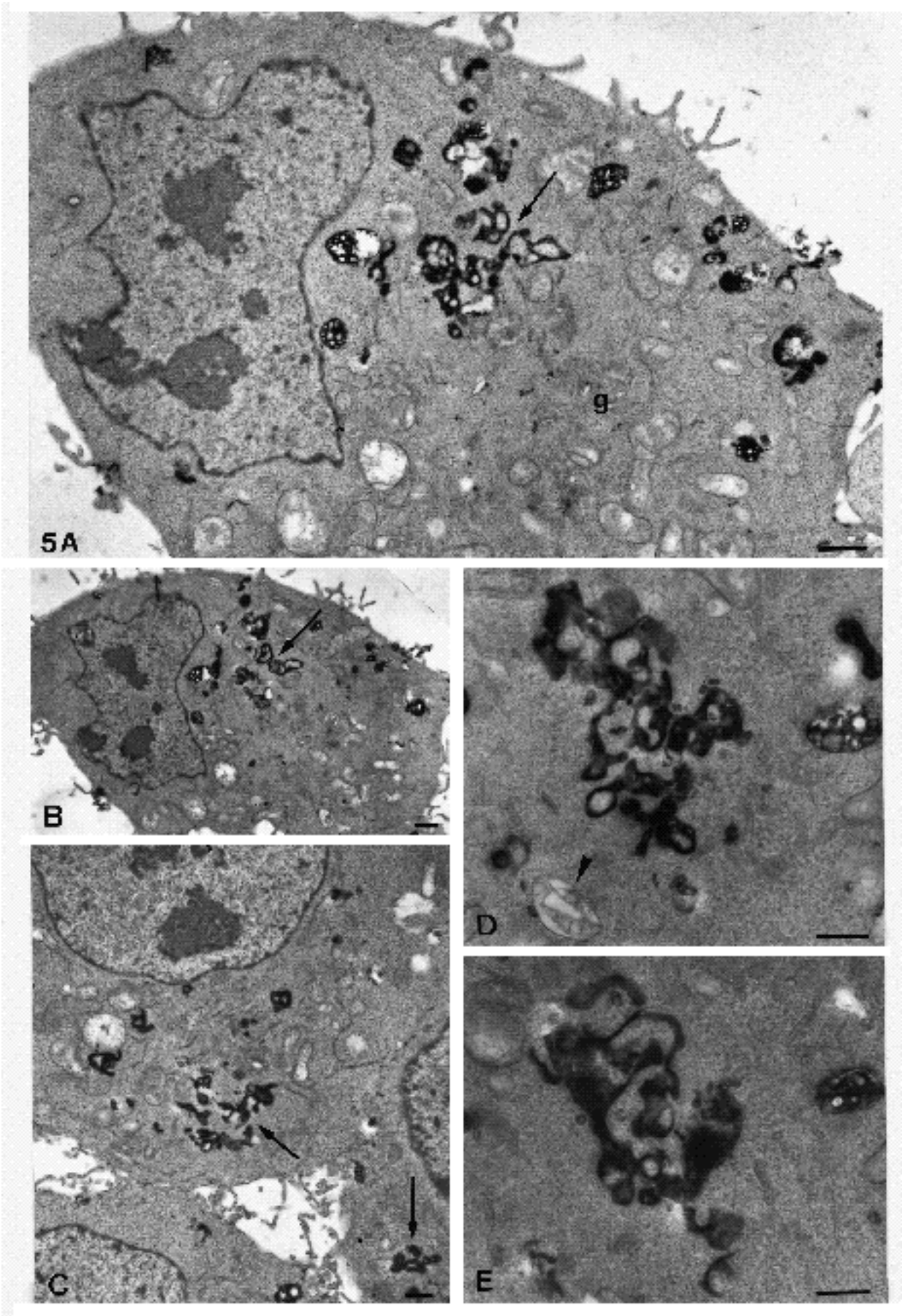
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Fig. 5. Semi-thick plastic sections of biotinylated cells incubated in the presence of avidin. Treatment of cells, processing and sectioning were performed as described in the legend to Fig. 4 but the incubation at 37°C was in the presence of 0.1 mg/ml avidin. HRP labels very large structures with a multivesicular content. These structures are not dispersed throughout the cell but are clustered in certain areas (arrows, compare with Fig. 4). (A,B) Low magnification serial section of cells with modified endosomes. (D and E) Higher-magnification views of a large HRP-labelled structure in two consecutive thick sections illustrating the three-dimensional complexity of the modified endosomes. Note the late endosome labelled with gold in D (arrowhead) which shows no HRP labelling. Bars: A-C, 1 μm; D,E, 0.5 μm.

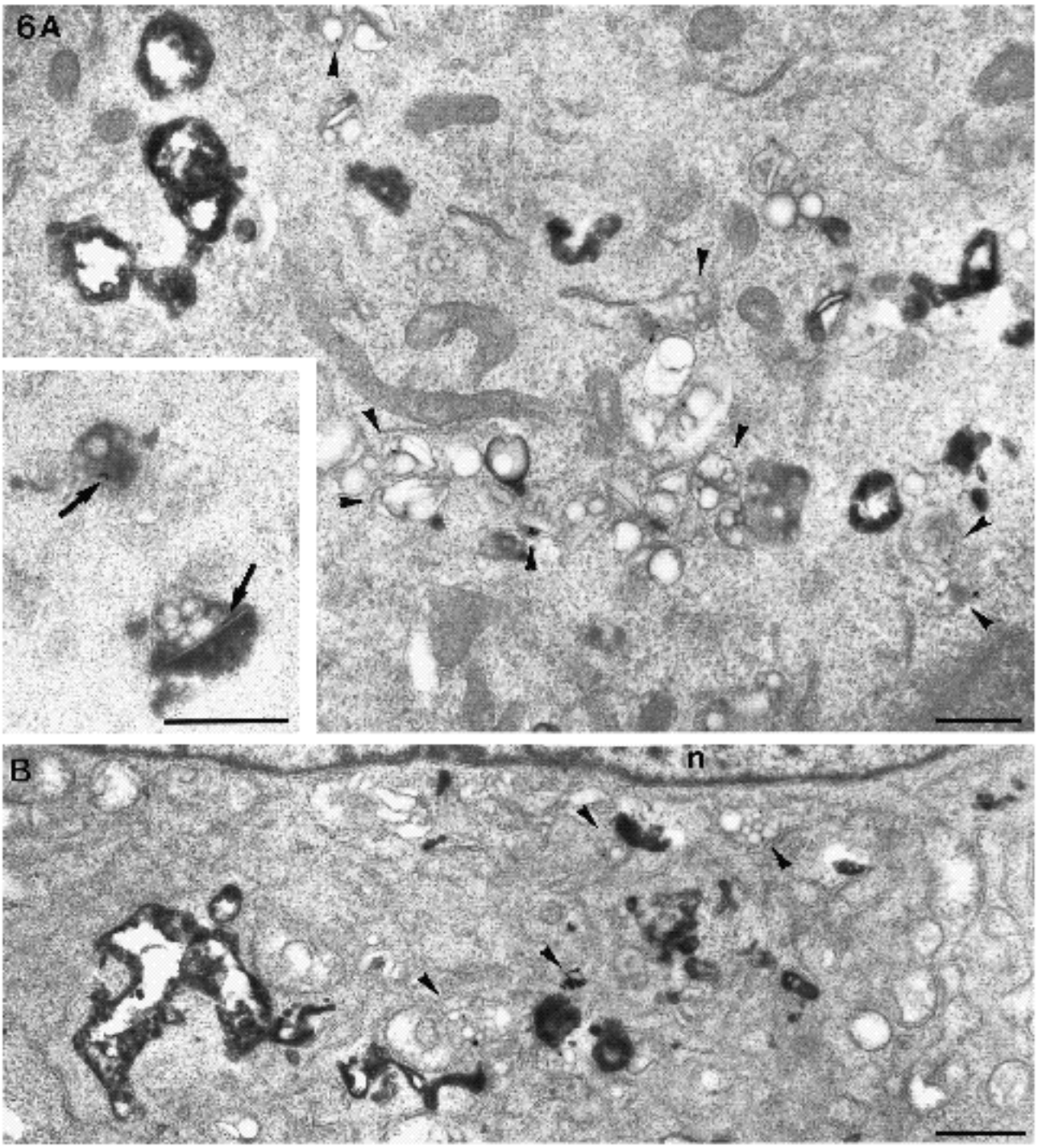

Fig. 6. Semi-thick sections of modified endosomes and late endosomes in biotin-avidin-treated cells. Cells were treated exactly as described in the legend to Fig. 5, but HRP was internalized for 10 min (A) or 30 min (B) at 37°C. The inset shows cells incubated with HRP for 10 min and chased for a further 40 min at 37°C. (A,B) HRP labels large endosomal structures but rarely is observed in late endocytic compartments which are labelled with gold (large arrowheads). However, after a 40 min chase (inset) HRP reaches late endosomes containing BSA-gold (arrows; the micrograph is printed lightly to allow visualization of both gold and HRP). n, nucleus. Bars, 1 μm.

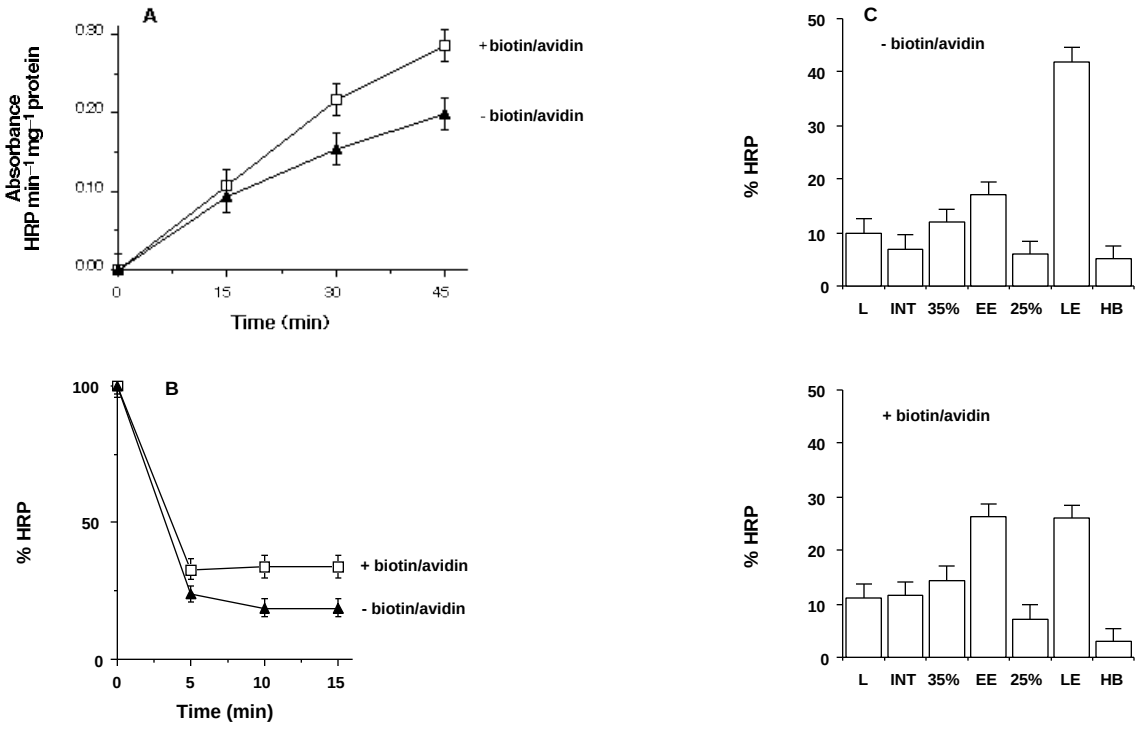

Fig. 7. Transport to/from early endosomes in biotin-avidin-treated cells. (A) Internalization. Monolayers were treated with biotin and avidin, as in Fig. 5 and reincubated at 37°C for the indicated times in the presence of 2 mg/ml HRP. The amount of cell-associated HRP was then quantified (+ biotin/avidin) and compared with control monolayers (− biotin/avidin). (B) Recycling. Early endosomes were labelled with HRP for 5 min at 37°C, washed extensively at ice temperature and then re-incubated at 37°C for the indicated times in HRP-free medium. As above, the amount of cell-associated HRP (+ biotin/avidin) is compared with that in control cells without the treatment (− biotin/avidin). Results are expressed as a percentage of the amount of HRP internalized in 5 min. (C) Transport to late endosomes. The early endosomes were labelled with HRP, as in B and then HRP was chased to late endosomes by incubation for 45 min at 37°C in HRP-free medium. The cells were then fractionated using a flotation step gradient (Gorvel et al., 1991) as described in Materials and methods. After the run, HRP was quantified in the different fractions: L, load; INT, interface load/35%; 35%, cushion of 35% sucrose; EE, position of early endosomes corresponding to the 35%/25% interface; 25%, cushion of 25% sucrose; LE, position of late endosomes corresponding to the uppermost portion of the 25% cushion; HB, homogenization buffer. The fractionation obtained using treated cells (+ biotin/avidin) is compared with that obtained using control cells (− biotin/avidin). The amount of HRP is expressed as a percentage of the total amount present in each gradient.

As shown in Fig. 7C, the percentage of HRP appearing in the late endosome fraction was reduced after the treatment (26%), when compared to control cells (42%), and more HRP co-fractionated with the early endosome fraction (26% versus 17%). These experiments suggest that transport can occur between early and late endosomes after the treatment, albeit with a reduced efficiency. Altogether, these data suggest that more HRP is accumulated in treated cells, because it is less efficiently recycled back to the external medium and transported towards late endosomes. As a consequence more HRP remains in early endosomes, in agreement with our observation that the size of the compartment is increased by the treatment (see Table 1).

Formation of modified endosomes is rab5-dependent

We have previously shown that the small GTP-binding protein rab5 is necessary for the fusion of early endosomes using a cell-free assay (Gorvel et al., 1991). In the experiments described here, we also showed that a mutant of rab5 with a single point mutation in the GTP binding domain, rab5Ile133, inhibited the fusion reaction. Recent in vivo experiments have indicated that overexpression of this mutant leads to a disruption of the early endosome organization (Bucci et al., 1992). Early endosomes then exhibit a morphological appearance quite different to that observed after the biotin-avidin treatment. Therefore, we tested whether the formation of endosomes would be prevented by overexpression of rab5Ile133. In these experiments, the endosomal content was labelled with HRP internalized for 30 min at 37°C, to guarantee labelling of the early endosomal compartment. As shown in Fig. 8, large early endosomal structures resembling modified endosomes were detected in cells overexpressing WT rab5 (Fig. 8A) but not rab5Ile133 (Fig. 8B,C). Endosomes then appeared as a collection of small tubules and vesicles scattered at the cell periphery. This typical disruption of early endosome organization after rab5Ile133 overexpression was very similar to that observed in control cells (not shown). These experiments suggest that a rab5-dependent step of membrane transport, presumably early endosome fusion, is required for the formation of modified endosomes.

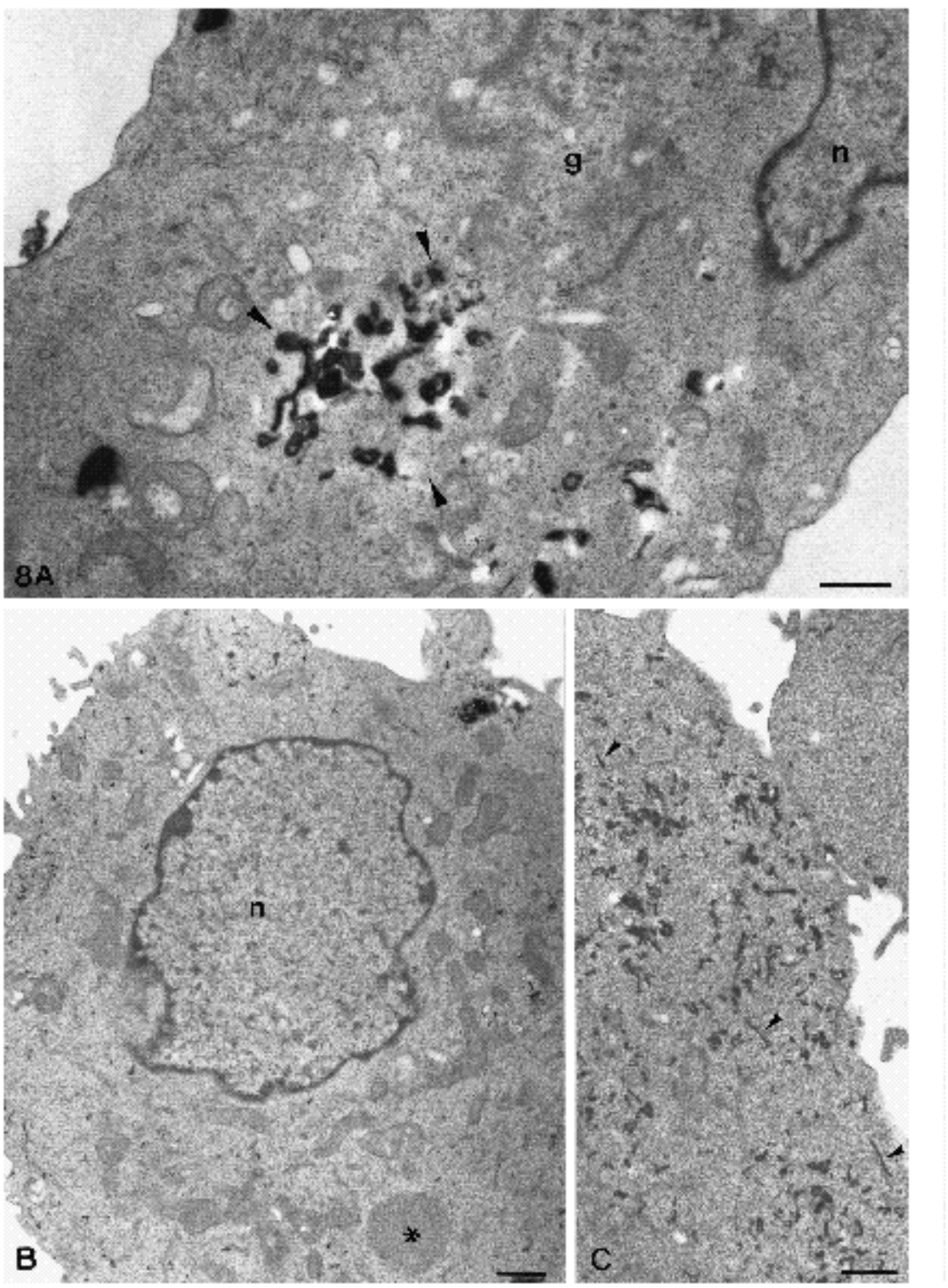

Fig. 8. Morphology of biotin-avidin-treated cells overexpressing rab5 or rab5Ile133. Cells were infected with recombinant vaccinia virus. Cells overexpressing rab5 (A) or rab5Ile133 (B,C)were then treated as described in the legend to Fig. 3, embedded in Epon and semi-thick sections were prepared. In cells overexpressing rab5 (A), HRP labels large endosomal structures (indicated by arrowheads) similar to those observed in biotin-avidin-treated cells (Fig. 5). In contrast, no such structures are seen in cells overexpressing rab5Ile133 (B,C) where HRP is distributed within small tubules (small arrowheads) and vesicles scattered throughout the cell. An asterisk indicates a vaccinia viral factory. n, nucleus. g, Golgi. Bars, 1 μm.

Discussion

In the present study, we have investigated the plasticity of early endosomes after cell surface biotinylation using a biotin derivative containing a disulphide bridge, followed by internalization of avidin. After this treatment, the morphological organization of early endosomes is dramatically changed. Early endosomes appear as large structures containing tubular, cisternal and vesicular regions, which are interconnected in a complex network that often extends over several micrometres. In addition, the lumen of the early endosomal structures appears more electron-dense and contains a complex organization of internal membranes. Since fewer peripheral tubules and small vesicles are then observed, we believe that individual early endosomal structures have become associated to generate these large modified endosomal structures. Morphometric studies suggested that the volume of this early endosome network increased relative to the total cell volume and the surface to volume ratio decreased. Despite these morphological changes, transport to and from early endosomes still occurs. Accumulation of a fluid-phase marker is increased whereas recycling and transport towards late endosomes are proportionally decreased when compared to untreated cells, consistent with the observation that the volume of early endosomes is then larger.

Our experiments suggest that these large structures arise from the cross-linking of the early endosome lumen mediated by the binding of avidin to internalized biotinylated proteins, since avidin in the absence of biotinylation does not lead to the formation of these structures. In contrast to the dramatic changes occurring in early endosomes, late endosomes remain indistinguishable from those observed in untreated cells and do not appear to be physically connected to this cross-linked, early endosomal network. With this approach, the structural organization of early endosomes could be modified without apparent changes at later stages of the pathway, or dramatic perturbations in the rate of transport to and from early endosomes.

Other protocols have been used to modifiy the structural organization of compartments of the endocytic pathway. In renal proximal tubules, high concentrations of ferritin decreased the surface density of apical tubules involved in membrane recycling to ≈ 50% of the control value in a reversible manner (Christensen, 1986). More recently, Heuser (1989) showed that acidification of macrophage cytoplasm resulted in the fragmentation of tubular lysosomes (Swanson et al., 1987; Knapp and Swanson, 1990) and their redistribution towards the cell periphery. We made a similar observation in epithelial MDCK cells, where late endosomes fragmented and redistributed to the basal surface on cytosolic acidification (Parton et al., 1991). These changes are rapidly reversible upon return to neutral pH, suggesting that late endosomes are highly dynamic. In fact, a recent study showed that tubular lysosomes in macrophages are labelled by antibodies to the late endosome-specific GTP-binding protein, rab7 (Chavrier et al., 1990), and may therefore be functionally equivalent to late endosomes in other cells (Rabinowitz et al., 1992). In addition, the latter study showed that internalization of wheat germ agglutinin caused the formation of an extensive and interconnected network of late endosomes/tubular lysosomes, without affecting early endosome organization. This observation adds further support to previous studies showing that tubular lysosomes/late endosomes are highly plastic (Knapp and Swanson, 1990).

Less is known about the plasticity of early endosomes in vivo. However, recent studies have shown that the fungal metabolite brefeldin A can change early endosomes both morphologically and functionally (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991). The fusion activity of early endosomes has been studied in vitro by several groups using different cell types (reviewed by Gruenberg and Howell, 1989) and some molecular aspects of this fusion event have now been studied in detail (Gruenberg et al., 1989; Tuomikoski et al., 1989; Diaz et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991). Because early endosomes are highly fusogenic in vitro, we have proposed that in vivo they may be organized in a dynamic network of elements that interact with each other via fusion and fission events, forming in effect a single compartment (Gruenberg and Howell, 1989). Recently, a complex tubular network of endosomes has been observed by videomicroscopy (Hopkins et al., 1990), and probably corresponds to the early endosome (Tooze and Hollinshead, 1991). This latter study also showed that the extent of this early endosome network varies significantly between cell types, and was not abundant in BHK cells, which is consistent with our observations.

In our studies, the formation of these complex endosomal structures occurs via avidin-mediated cross-linking of proteins that were originally on the plasma membrane. Since early endosomes then appear interconnected, we propose that they are still free to undergo lateral fusion with each other, as observed in vitro, but that subsequent fission events, that must occur to maintain the size and function of individual early endosomal elements, are prevented. This proposal is consistent with our observation that the formation of large, cross-linked structures does not occur after overexpression of a rab5 mutant that inhibits early endosome fusion in vitro (Gorvel et al., 1991). Early endosomes then appear fragmented, consisting of small vesicular and tubular elements scattered at the cell periphery. Clearly, only a fraction of the biotinylated proteins participates in the cross-linking process, since streptavidin-gold binding sites are still abundant in these large structures. It is, however, not clear whether the changes are caused by the formation of an intraluminal meshwork betweeen different biotinylated molecules or whether they are due to the specific immobilization of putative components required for early endosome fission.

Our data indicate that the organization, size and shape of early endosomes can be changed dramatically, without causing an arrest of endocytic membrane traffic. This shows that early endosomes, like late endosomes and tubular lysosomes, are highly plastic organelles and suggests that in vivo they are connected to each other by fusion and fission events.

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