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Mitotic Golgi clusters are not tubular endosomes

Marc Pypaert[1,*], Tommy Nilsson[1], Eric G. Berger[2] and Graham Warren[1,†]

[1]Cell Biology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
[2]Physiologisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland

*Present address: Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland
†Author for correspondence

SUMMARY

HeLa cells were incubated with 15 nm BSA-gold for 1 or 2 hours to mark the endocytic pathway and mitotic cells were then isolated by shake-off. Thin, frozen sections were labelled with antibodies against two resident Golgi markers, β-(1,4)-galactosyltransferase and *N*-acetylglucosaminyltransferase I. Detection of the latter was aided by the use of a HeLa cell line stably expressing a myc-tagged version of the endogenous protein. The secondary antibodies were coupled to either 5 or 10 nm gold so that the distribution of each of the three markers could be followed. Qualitative and quantitative studies showed that there were two populations of clusters, those described by us earlier and termed Golgi clusters (Lucocq et al. (1987) *J. Cell Biol.* 104, 865-874), containing either or both Golgi markers, and clusters of tubular endosomes containing BSA-gold. There was very little overlap showing that Golgi clusters cannot be tubular endosomes as concluded by Tooze and Hollinshead (1992) *Eur. J. Cell Biol.* 58, 228-242.

Key words: Golgi, mitosis, endosomes

INTRODUCTION

At the onset of mitosis in animal cells, the Golgi apparatus fragments and vesiculates, yielding several hundred Golgi clusters (Lucocq and Warren, 1987). These shed vesicles, which become dispersed throughout the mitotic cell cytoplasm (Lucocq et al., 1989). Providing the mother cell is then divided into two equally sized daughters (Rappaport, 1986), nearly equal partitioning of the Golgi will occur by a stochastic process (Birky, 1983; Warren, 1985, 1993). Re-assembly takes place during telophase by essentially the reverse process (Lucocq et al., 1989).

Golgi clusters, as their name implies, are groups of vesicles and tubules set in a dense matrix and bounded by the transitional elements of the endoplasmic reticulum (ER), sometimes on all sides. These morphological criteria were based on cytochemical and immunocytochemical studies used to identify mitotic Golgi membranes. Cluster vesicles could be stained for thiamine pyrophosphatase (TPPase) (Lucocq et al., 1987), a *trans*-Golgi marker (Novikoff, 1976), impregnated with osmium (Lucocq and Warren, 1987), a *cis*-Golgi marker (Friend and Murray, 1965), and labelled with antibodies to β-(1,4)-galactosyltransferase (GalT) (Lucocq et al., 1987, 1989), a resident protein of *trans*-Golgi cisternae (Roth and Berger, 1982).

The role of the clusters in the Golgi division pathway was defined by several studies. The disappearance of Golgi stacks at the onset of mitosis was accompanied by the appearance of clusters (Lucocq et al., 1987). Metaphase cells contained 10 to 300 clusters (Lucocq and Warren, 1987), those with the fewest having the most free vesicles and vice versa (Lucocq et al., 1989). This strongly suggested that clusters gave rise to free vesicles. During telophase the clusters grew by accretion of vesicles, which then fused to form discrete stacks (Lucocq et al., 1989). All of these data placed clusters firmly on the Golgi division pathway.

Despite these data, a recent paper casts doubt on their identity. Tooze and Hollinshead (1992) labelled the endocytic pathway of HeLa cells with either horseradish peroxidase (HRP) or BSA-gold and found that structures resembling Golgi clusters were labelled in mitotic cells. The HRP labelling extended to almost all of the vesicles in any one cluster, and to essentially all of the clusters. This led them to conclude that Golgi clusters are not derived from the Golgi apparatus but are, in fact, clustered tubular endosomes. This conclusion was based entirely on a morphological examination of the sections. No attempt was made to apply any of the more rigorous cytochemical and quantitative immunocytochemical criteria that we had established as necessary for the proper identification of Golgi clusters. Nevertheless, the apparent absence of clusters not labelled by HRP was puzzling and warranted further investigation.

In this paper we have labelled the endocytic pathway with BSA-gold and used antibodies to two Golgi markers. Since some doubt was cast on our original use of GalT we have localised another resident Golgi marker, *N*-acetylglu-

cosaminyltransferase I (NAGT I). These triple-labelling experiments gave unequivocal results, showing that there are two populations of clusters. This leads, inescapably, to the conclusion that Golgi clusters are not tubular endosomes.

MATERIALS AND METHODS

Materials

Fraction V bovine serum albumin (BSA) (Boehringer Mannheim) was conjugated to 15 nm colloidal gold as described by Slot and Geuze (1985). Unless otherwise stated, all other reagents were obtained from Sigma Chemical Co. or BDH.

Cell culture and incubation with BSA-gold

The HeLa cell line stably expressing a myc-tagged version of NAGT I (Nilsson et al., 1993) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Northumbria Biologicals Limited) supplemented with 10% foetal calf serum, penicillin, streptomycin and 200 μg/ml Geneticin (G-418 sulphate) (Gibco). Cells were synchronised using a double-thymidine block (Bootsma et al., 1964) and 8 hours after release from the second thymidine block culture medium containing BSA-gold ($A_{520} \approx 50$) was added. Following one or two hours of incubation, mitotic cells were shaken off (Klevecz, 1975) and washed extensively with culture medium before fixation.

Immunoelectron microscopy

Mitotic cells were fixed for 1 h at room temperature (RT) with 0.5% (w/v) glutaraldehyde (Fluka BioChemika, Glossop, England) in 0.2 M Pipes (piperazine-*N*,*N'*-bis-2-ethanesulfonic acid), pH 7.2, then washed three times in the same buffer and pelleted at 13,000 revs/min for 10 min at RT in an Eppendorf centrifuge. Pellets were infused with a solution of 2.1 M sucrose in Dulbecco's PBS, pH 7.4, for 30 min at RT, then quickly frozen in liquid nitrogen.

Frozen sections were cut, labelled with polyclonal antibodies against GalT (Watzele et al., 1991) and the monoclonal antibody 9E10 against c-*myc* (Evan et al., 1985), followed by appropriate secondary antibodies conjugated to either 5 or 10 nm gold, and finally infiltrated with epoxy resin as described previously (Nilsson et al., 1993). Sections were examined in a Philips CM10 electron microscope.

Stereological analysis

For each condition tested, about 10 mitotic cells were sampled, which, from the arrangement of their chromosomes, were in either metaphase or prometaphase (see Lucocq et al., 1987). All the clusters present were photographed at a magnification of 28,500. Clusters were defined as any group containing membrane profiles of five or more vesicles and/or tubules. To be included in the group any one of these structures had to be within a certain distance of its nearest neighbour. This distance was either the diameter of the smallest vesicle in the cluster or the width of the smallest tubule. This was typically about 50 nm. In early experiments there was no restriction on the size of the structures. In later experiments the counting was restricted to clusters containing structures with a diameter or width no greater than 120 nm.

The surface area of clusters was determined by point counting using a grid with 4.3 mm line spacings. Counting was done directly on the negatives. The surface area in μm^2 was 0.026 × the number of points over the cluster area. The number of each size of gold particle over each cluster was counted so that the density (gold particles/μm^2 of cluster surface area) could be worked out. Background densities for both GalT and NAGT I were subtracted. These were calculated from the labelling density over 10 mitochondrial profiles.

RESULTS

Labelling of interphase HeLa cells

Double-labelling of the Golgi apparatus was made possible by a HeLa cell line stably expressing a slightly modified version of the resident enzyme, NAGT I (Nilsson et al., 1993). The modification, to the C terminus (the luminal domain), was the addition of a myc epitope, recognised by the high-affinity monoclonal antibody, 9E10 (Evan et al., 1985).

Thin, frozen sections were labelled with polyclonal antibodies specific for the polypeptide chain of GalT (Watzele et al., 1991) and the 9E10 monoclonal antibody followed, respectively, by anti-rabbit and anti-mouse secondary antibodies conjugated to different sizes of gold particles. As shown in Fig. 1, GalT was localised to two cisternae on one side of the stack, which we had earlier assigned as the

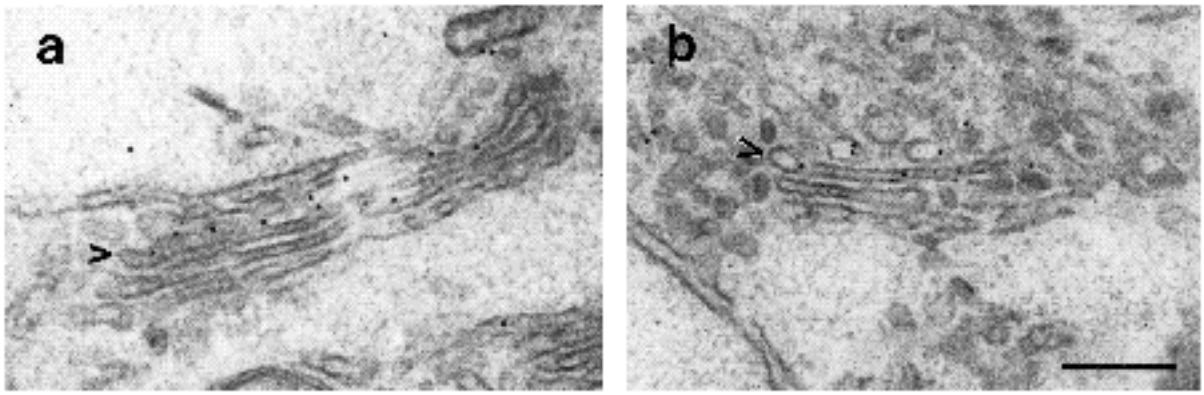

Fig. 1. Double-labelling of the Golgi apparatus in interphase HeLa cells. Thin, frozen sections of the stable HeLa cell line were labelled with both polyclonal anti-GalT and 9E10 antibodies followed by appropriate secondary antibodies coupled to different sizes of gold (GalT, 10 nm gold; NAGT I, 5 nm gold). The *trans* cisterna (open arrowhead in both a and b) was defined as the last continuous cisterna containing GalT (see Nilsson et al., 1993). Note that GalT is mostly present in both the *trans* cisterna and the *trans*-Golgi network (TGN), whereas NAGT I is in both the *medial* and *trans* cisternae and very occasionally in the TGN. Bar, 0.25 μm.

trans cisterna and the *trans*-Golgi network (TGN). NAGT I was localised to the *medial* and *trans* cisternae, overlapping the distribution of GalT (see Nilsson et al., 1993). Only Golgi membranes were labelled. There was no significant labelling of the plasma membrane or any other organelle.

Labelling of mitotic HeLa cells

Since mitotic cells do not endocytose (Berlin and Oliver, 1980), BSA-gold had to be introduced into interphase cells before they entered mitosis. This was carried out as described by Tooze and Hollinshead (1992) using a 1- or 2-hour pre-incubation. By growing and synchronising the cells in a roller bottle it was possible to isolate a highly enriched population of mitotic cells by rapid rotation of the bottle. Shake-off is the mildest method available for the production of mitotic cells and yields all stages with the exception of prophase (Klevecz, 1975). The work described here focuses on prometaphase and metaphase cells, which are easily distinguished from all other stages.

Mitotic cells were fixed and prepared for labelling with both anti-GalT and 9E10 antibodies. Secondary antibodies were conjugated to either 5 nm (NAGT I) or 10 nm (GalT) gold, which, together with the use of 15 nm BSA-gold, permitted triple-labelling studies.

A representative selection of Golgi clusters is shown in Fig. 2, chosen for their content of GalT and NAGT I. Almost every cluster contained both markers, especially the larger ones, and those individual membrane profiles that were labelled appeared to contain either or both markers. This is consistent with the overlapping distribution seen in interphase cells (Fig. 1; and Nilsson et al., 1993). Membrane profiles containing neither marker were presumably derived from the *cis* cisterna or *cis*-Golgi network (CGN), though some could represent unlabelled *medial* and *trans*-Golgi membranes. These clusters met all of the morphological criteria previously established for the parental HeLa cell line on the basis of GalT labelling alone (Lucocq et al., 1987). They comprised both vesicular and tubular profiles with a diameter or width of around 50 nm and they were often found in association with elements of the ER (Fig. 2c and e), though whether these were derived from the transitional element region was not always clear.

Fig. 3 presents a selection of clustered tubular endosomes, chosen for their content of BSA-gold. The structures were far more heterogeneous than Golgi clusters and fell into two overlapping groups. One group comprised collections of vacuoles with tubular extensions (Fig. 3a, c, d and e). The number of vacuoles in the cluster varied and this group least resembled Golgi clusters morphologically. The other group comprised collections of vesicles and tubules that did resemble Golgi clusters (cf. Fig. 3b, f with Fig. 2e).

Comparison of Golgi clusters (Fig. 2) with clustered tubular endosomes (Fig. 3) showed that there was little overlap in their content of markers. Golgi clusters contained either or both Golgi markers but very little BSA-gold. Where BSA-gold was observed it was almost always within membrane profiles at the periphery of the cluster that did not contain either Golgi marker (data not shown). Clustered tubular endosomes contained BSA-gold but very little of either Golgi marker. When Golgi markers were present they were mostly found in peripheral membranes that did not contain BSA-gold (open arrowhead, Fig. 3d).

Quantification of labelling

These qualitative observations were confirmed by quantifying the distributions of the three markers between the two types of cluster. The area of each cluster profile was measured and the density of each of the three sizes of gold particle was calculated. Fifty-six clusters were sampled at random and each gold size plotted against each of the others. As shown in Fig. 4A, a plot of GalT against NAGT I yielded points scattered throughout the body of the graph, consistent with both markers being in the same population of clusters. The overall distribution of densities was skewed more towards the NAGT I axis because labelling for this marker was higher. The variation in density reflected the heterogeneity of labelling within the clusters (Fig. 2) and their size, which ranged from groups of five profiles to several hundred. The smallest clusters were found, as expected, to show the widest variations in density of both markers and often contained only one of the Golgi markers.

A plot of BSA-gold against GalT (Fig. 4B), NAGT I (Fig. 4C) or both (Fig. 5A) gave a completely different distribution. The great majority of the points lay on or very near to one or other of the axes. In other words, those clusters containing BSA-gold did not contain Golgi markers and vice versa, showing that there were two populations of clusters. Those data points that did not lie on or very near either axis almost always represented clusters where the minority label was only a few gold particles. Almost all of these were endosomes containing trace Golgi markers.

To show that BSA-gold had reached all endosomal compartments before the cells had entered mitosis, the pre-incubation time was increased from 1 to 2 hours. Comparison of Fig. 5A and B shows that this had no effect on the distributions.

As noted earlier, many of the clustered tubular endosomes did not resemble Golgi clusters morphologically, largely because of the presence of large vacuoles with tubular extensions (Fig. 3a, c, d and e). Though not stated explicitly in our earlier work, we did apply a size limit to the vesicles and tubules in the Golgi clusters and this would have excluded most of the endosomal clusters. Some vesicles in the Golgi clusters described earlier had diameters up to 250 nm but these were mostly the consequence of embedding samples in Lowicryl and they did not appear to be a significant population when thin, frozen sections were used. In any event these larger vesicles were much smaller than the larger endosomes illustrated in Fig. 3. The data presented in Fig. 5 were re-scored using an upper limit of 120 nm for the diameter or width of a membrane profile and the results are presented in Fig. 6. Most of the tubular endosomes were no longer counted, which explains why most of the data points now lie on the Golgi marker axis. Changing the pre-incubation time with BSA-gold from 1 to 2 hours did not affect the distribution (cf. Fig. 6A and B). From these data we can conclude that 75 to 85% of the clusters defined by morphological criteria alone were derived from the Golgi apparatus, the rest being tubular endosomes. This agrees well with our earlier estimates of

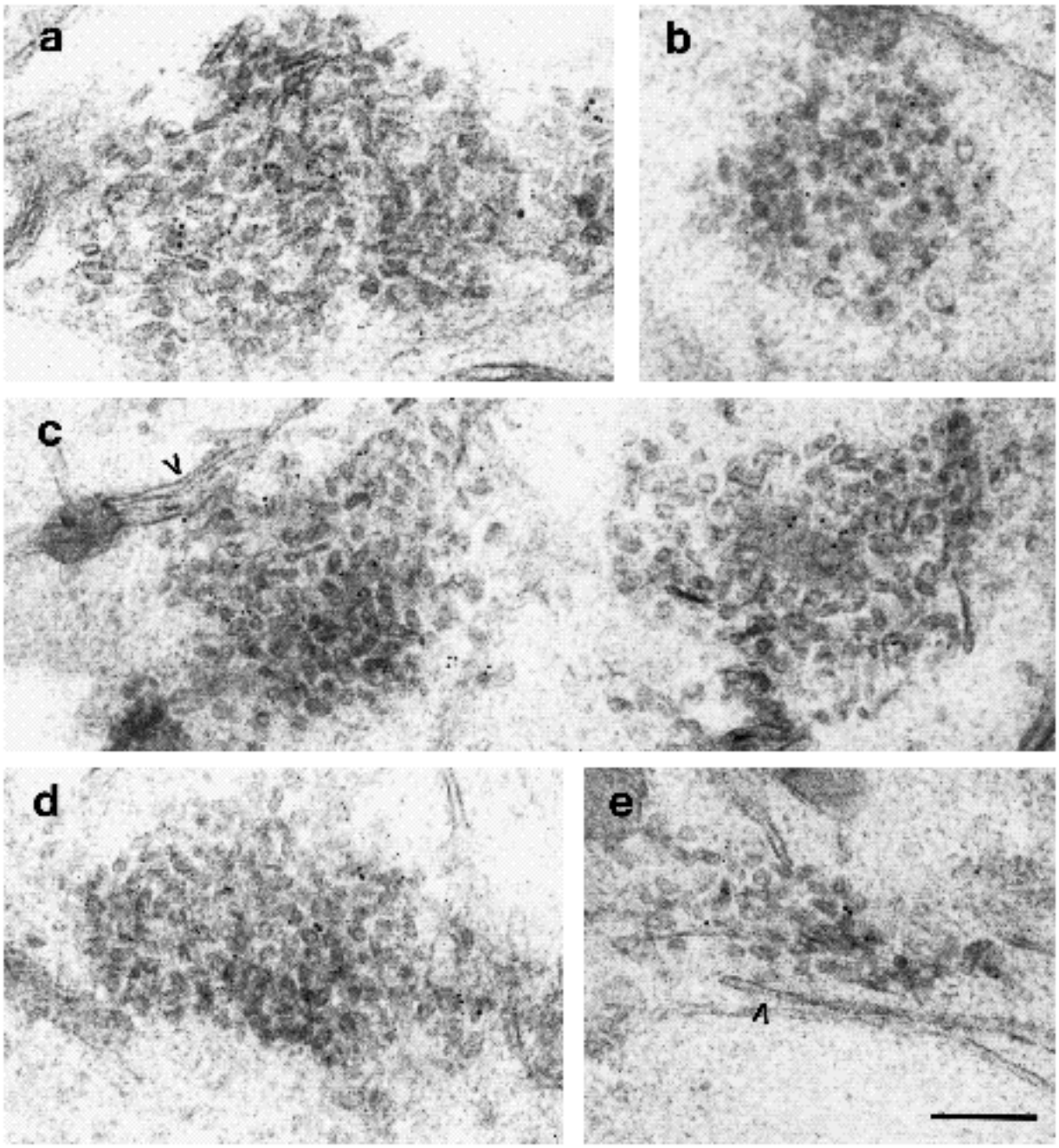

Fig. 2. Double-labelling of Golgi clusters in mitotic HeLa cells. Interphase cells were pre-incubated with (b-e) or without (a) 15 nm BSA-gold for 2 hours before cells that had entered mitosis were harvested by shake-off. Sections were then labelled for both GalT (10 nm gold) and NAGT I (5 nm gold). In some cases the clusters are adjacent to elements of the ER (open arrowheads in c and e). Note the total absence of BSA-gold in the clusters in b-e. Bar, 0.25 μm.

61 to 83%, arrived at using only GalT as the marker (Lucocq et al., 1989).

DISCUSSION

Using the morphological criteria we had established for Golgi clusters, Tooze and Hollinshead (1992) found that most if not all such structures in mitotic HeLa cells could be labelled with endocytosed HRP. They further showed that pre-treatment with brefeldin A (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989), causing fusion of Golgi membranes with the ER, had no effect on the labelling or frequency of these structures in mitotic cells, arguing that they were not derived from Golgi membranes. They suggested that free vesicles were the mitotic form of the Golgi apparatus (Lucocq et al., 1989), derived not from shedding clusters but directly from the Golgi apparatus. Their conclusion forced them to re-examine the criteria we had used to define Golgi clusters.

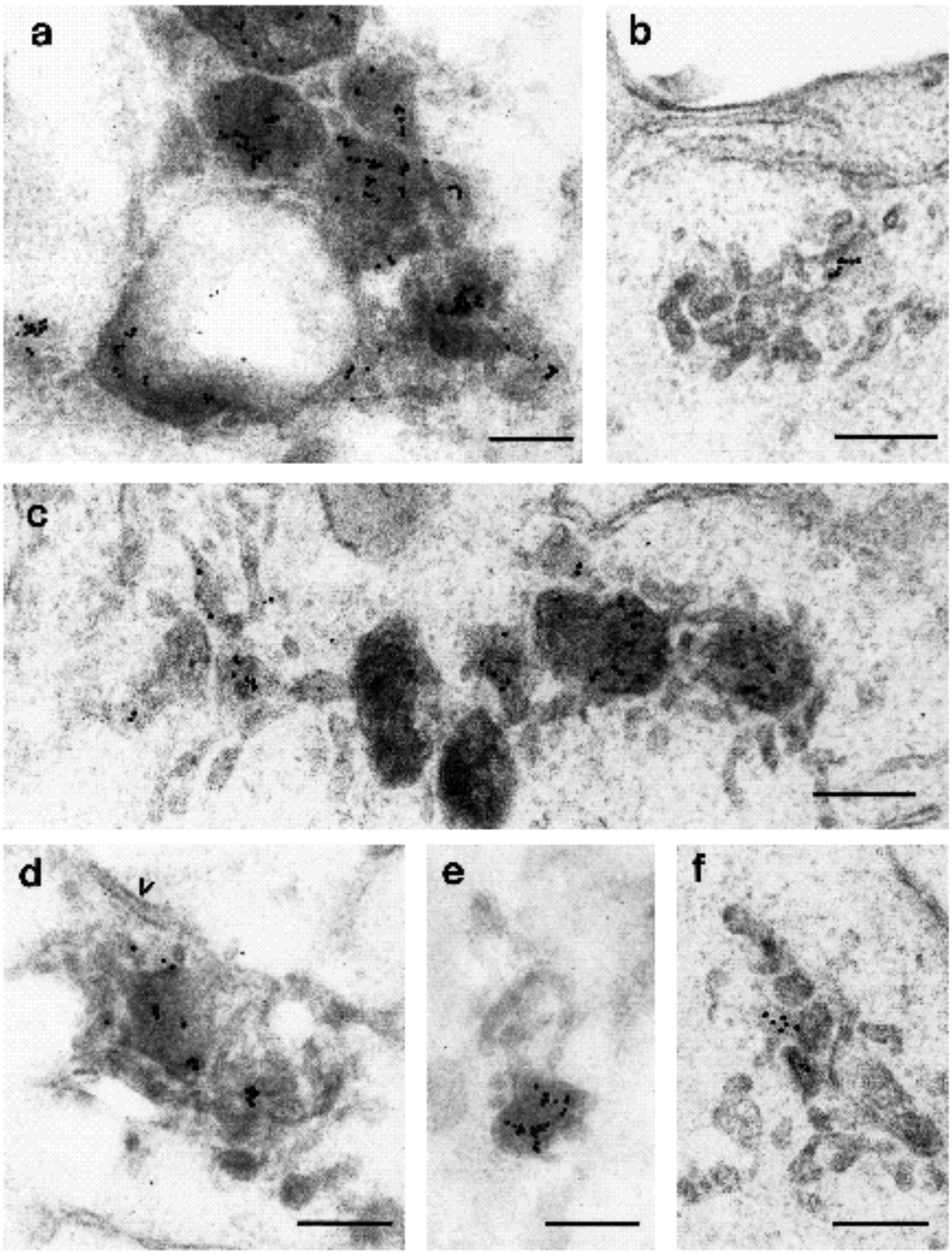

Fig. 3. BSA-gold labelling of tubular endosomes in mitotic HeLa cells. Samples were the same as in Fig. 2 (b, c, d and f) or were prepared from cells that had been pre-incubated with 15 nm BSA-gold for 1 hour (a, e) before the mitotic cells were harvested. Sections were labelled for both GalT (10 nm gold) and NAGT I (5 nm gold). A selection of clusters containing BSA-gold is shown ranging from varying numbers of grouped vacuolar structures with tubular extensions (a, c, d, e) to smaller tubular clusters containing vesicles (b, f). NAGT I and GalT were observed only occasionally (open arrowhead in d pointing to NAGT I-labelled element). Bar, 0.25 μm.

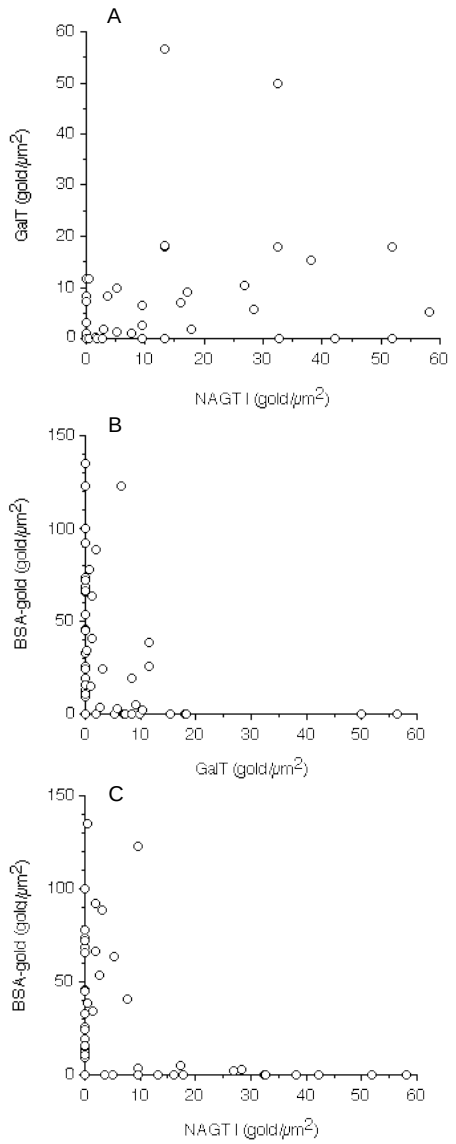

Fig. 4. Quantification of labelling over the clusters. Interphase cells were allowed to endocytose 15 nm BSA-gold for 1 hour before mitotic cells were harvested and sections labelled for both GalT (10 nm gold) and NAGT I (5 nm gold). The numbers of each size of gold for each cluster were divided by the area of the cluster profile. The density of each marker was plotted against each of the others after subtraction of background (GalT, 1.2 gold particles/μm^2; NAGT I, 5.9 gold particles/μm^2).

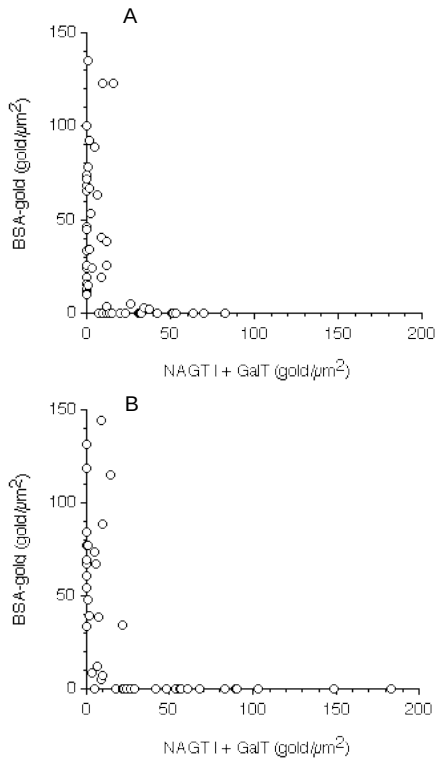

Fig. 5. Effect of pre-incubation time with BSA-gold on the distribution of the three markers. Interphase cells were allowed to endocytose 15 nm BSA-gold for 1 (A) or 2 (B) hours before mitotic cells were harvested and sections labelled for both GalT (10nm gold) and NAGT I (5 nm gold). The density of BSA-gold over each cluster was plotted against that for the sum of GalT and NAGT I after background subtraction (A: GalT, 1.2 gold particles/μm^2; NAGT I, 5.9 gold particles/μm^2; B: GalT, 4.0 gold particles/μm^2; NAGT I, 5.4 gold particles/μm^2). The abscissae are set to the same scale for easier comparison.

One criterion was the associated ER which bounded the clusters, often on all sides. This criterion was the consequence of identifying cluster vesicles following cytochemical and immunocytochemical studies. Tooze and Hollinshead (1992) argued that such an association could be fortuitous, the consequence of crowding in the peripheral mitotic cytoplasm where most of the clusters are found. What they omitted to note was that the ER associated with Golgi clusters is mostly derived from the transitional element region, which is characterised by "budding" profiles. This is the small region of the ER most commonly found in association with the interphase Golgi and thought to function in transport of newly assembled proteins and lipids to the Golgi apparatus (Palade, 1975). Such an association with Golgi clusters could hardly, therefore, be fortuitous.

They also argued that staining for TPPase was not a good criterion because staining is sporadic and not seen by all workers. But TPPase is a specific Golgi marker. In fact, its introduction by Novikoff (Novikoff and Goldfischer, 1961) finally convinced workers in the field that the Golgi apparatus was present in all cells. It is specific for *trans*-Golgi membranes (Novikoff, 1976) and no others, so if staining is seen then it is good evidence for the presence of *trans*-Golgi components.

They argued further that osmium impregnation could not be used to identify *cis*-Golgi membranes because other organelles, notably the ER and lysosomes, were also, on occasion, stained. This is true, but there have been no reports showing that endosomes are stained, so even if these data do not identify *cis*-Golgi membranes unequivocally they do strongly suggest that they are not endosomes.

Lastly, they argued that immuno-labelling with antibodies to GalT could be artefactual. This enzyme is a well-characterised marker for *trans*-Golgi cisternae, though care must be taken, as we did, to use antibodies that recognise

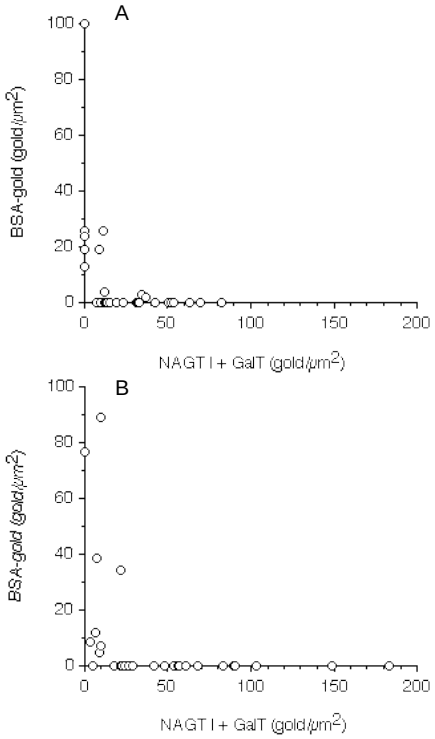

Fig. 6. Effect of imposing an upper limit to vesicle or tubule size on the distribution of the three markers. The data presented in Fig. 5 were re-scored to include only those clusters in which the profile diameter or width was ≤ 120 nm. BSA-gold was endocytosed for either 1 (A) or 2 (B) hours. The abscissae are set to the same scale for easier comparison.

the protein and not the associated oligosaccharides. But there are reports that GalT is present on the surface of some cells (Shur, 1989), though not on HeLa cells as we were careful to investigate (Lucocq et al., 1987). Nevertheless, given that GalT can be redistributed to the cell surface in some cells it is conceivable that it could do so during mitosis in HeLa cells and somehow end up in tubular endosomes. For this reason we looked at another Golgi marker, NAGT I, which has only ever been found in the Golgi apparatus (Dunphy et al., 1985). We were able to do these experiments because of a HeLa cell line that we had generated, which stably expresses a myc-tagged version of the endogenous protein (Nilsson et al., 1993). A number of criteria showed that this protein behaved in the same way as the endogenous protein and allowed us, for the first time, to double-label the Golgi stack. This showed that NAGT I overlapped the distribution of GalT, an unexpected result, since NAGT I had been thought to be restricted to medial cisternae (Dunphy et al., 1985).

The results using NAGT I confirmed and extended our identification of Golgi clusters. Cluster vesicles contained one, both or neither of the two markers, as expected from their distribution in interphase Golgi stacks. This suggests that cisternal membranes retain their identity during vesiculation, as predicted by our hypothesis (Warren, 1985, 1989, 1993). Vesiculation is suggested to be the consequence of continued budding of transport vesicles at the onset of mitosis which can no longer fuse with the next cisterna in the stack. These distributions also confirm the identity of Golgi clusters, further validating our earlier criteria.

To eliminate the possibility that Golgi and endosomal membranes become mixed during mitosis, we carried out triple-labelling experiments using endocytosed BSA-gold as the marker for the endocytic pathway. Tooze and Hollinshead state that they obtained similar results using either BSA-gold or HRP, though most of the work they presented utilised the latter marker. We chose to look at BSA-gold because its level could be quantified. The results were unequivocal. BSA-gold was present in one population whereas GalT and NAGT I were present in another. There was very little overlap and that which did exist was most often in membranes on the periphery of the clusters. Golgi clusters are not tubular endosomes.

Despite the unequivocal nature of these results they do not explain those obtained by Tooze and Hollinshead (1992). Since they relied only on morphological criteria it is possible that they simply missed the Golgi clusters. They used thicker sections, which would reduce the probability of finding them, and they did not quantify their results except to state that only about 2% of the clusters were unlabelled. Their means of estimating this value were not described but we would estimate that about 50% of all clusters, counted without regard to the size of the profile, would be Golgi clusters. It is difficult to believe that so many would have been missed, so the possibility of artefactual labelling must be considered. The enzymatic activity of the HRP used was up to 100-fold higher than that typically used to label the endocytic pathway. This level was needed to visualise narrow tubular endosomes. These levels did not label the Golgi apparatus in interphase cells, probably because the net flow of material is outwards. However, membrane traffic stops at the onset of mitosis and the smallest leak backwards could have stained the Golgi clusters. Their argument against this possibility is that the reassembling Golgi in telophase cells did not contain any HRP. But intra-Golgi transport resumes as the Golgi is being reassembled (Collins and Warren, 1992) and transport from the *trans*-Golgi network (TGN) to the cell surface is the one step on the exocytic pathway that is not inhibited during mitosis (Kreiner and Moore, 1990). This outward flow could have drained the reassembling Golgi of any HRP giving the negative results observed by Tooze and Hollinshead. If high levels of HRP are needed to stain Golgi clusters this would also explain our earlier, preliminary observations showing that more normal levels of HRP did not stain them (Lucocq et al., 1988).

This suggestion does not explain their experiment where brefeldin A was used to send the Golgi back to the ER before the cells entered mitosis. They found that the number of clusters did not change. Unfortunately, it would seem that this was a qualitative statement, not a quantitative measure and the work described in this paper emphasises yet again the pitfalls of qualitative methods and the value of quantitative immuno-cytochemistry.

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