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The membrane spanning domain of β-1,4-galactosyltransferase specifies *trans* Golgi localization

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Chimeric cDNAs were constructed so as to generate hybrid proteins in which different parts of the N-terminal domain of the human invariant chain were replaced by equivalent sequences from the *trans* Golgi resident enzyme, β-1,4-galactosyltransferase. The cytoplasmic and membrane spanning domains of galactosyltransferase were found to be sufficient to retain all of the hybrid invariant chain in *trans* Golgi cisternae as judged by indirect immunofluorescence, treatment with brefeldin A and immuno-electron microscopy. As few as ten amino acids corresponding to the lumenal half of the membrane spanning domain of the Golgi enzyme sufficed to localize most of the hybrid invariant chain to the *trans* cisternae. A cytoplasmic domain was necessary for complete retention as assessed by flow cytofluorometry but could be provided either by galactosyltransferase or by invariant chain. This suggests that the cytoplasmic domain plays a role accessory to the membrane spanning domain, the latter mediating compartmental specificity.

Key words: compartmentalization/β 1,4-galactosyltransferase/Golgi/invariant chain/retention

Introduction

Proteins destined for the exocytic pathway are directed into the endoplasmic reticulum (ER) by an N-terminal signal peptide (Blobel and Dobberstein, 1975; von Heijne, 1985). Following interaction with the signal sequence receptor (Wiedmann *et al.*, 1987; Görlich *et al.*, 1990), the polypeptide is translocated into the ER lumen where the signal peptide is removed and the polypeptide chain subjected to further post-translational modifications (reviewed by Rose and Doms, 1988; Freedman, 1989; Hurtley and Helenius, 1989). However, for some proteins the signal peptide remains uncleaved and is utilized to anchor the polypeptide in the membrane. Depending on the balance between charges on the cytoplasmic and lumenal residues adjacent to the membrane spanning domain, the remainder of the polypeptide may undergo translocation (Szczesna-Skorupa and Kemper, 1989; Nilsson and von Heijne, 1990; Parks and Lamb, 1991). If translocated, the polypeptide chain exhibits inverted or type II topology with its N-terminus projected into the cytoplasm as opposed to a type I protein which has its N-terminus located extracellularly and is anchored in the membrane as a result of an internal stop transfer sequence (see Wickner and Lodish, 1985 for review). Following translocation into the ER, proteins are thought to move towards the plasma membrane by default unless held back by structural motifs (see Pfeffer and Rothman, 1987 for review). In the case of ER residents, such motifs have been identified for both lumenal and transmembrane proteins (Munro and Pelham, 1987; Pääbo *et al.*, 1987; Stirzaker and Both, 1989; Kuroki *et al.*, 1989). When these motifs are transplanted onto reporter proteins that would otherwise be transported, they now confer ER residence (Munro and Pelham, 1987; Nilsson *et al.*, 1989). This does not rule out the possibility, however, that other parts of a resident protein might be of importance for retention.

While both type I and II proteins can be found among residents of the ER, all resident proteins of the Golgi stack identified so far belong to the type II category (reviewed by Paulson and Colley, 1989). One of the most extensively characterized Golgi residents is the β-1,4-galactosyltransferase (GT) (EC 2.4.1.22) normally present in the *trans*-most cisternae of the Golgi stack (reviewed by Strous, 1986). Since a proteolytically derived soluble form of GT, consisting of almost the entire ectodomain, can be found extracellularly *in vivo*, it has been suggested that the information responsible for Golgi localization most likely resides in the N-terminal portion of this molecule (Paulson and Colley, 1989). Another type II protein, the human invariant chain (Ii), usually found associated with the major histocompatibility complex (MHC) class II antigens, seems to be similar to GT in having targeting information in this region. Two major forms of Ii have recently been characterized in detail, Iip33 and Iip31. While the former is a resident of the ER (Lotteau *et al.*, 1990), the latter chaperones newly synthesized MHC class II α and β chains through the Golgi to an endocytic compartment. Based on N-terminal deletions of the Ii cytoplasmic domain, it has been suggested that sorting information resides within this domain (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990).

To test whether information for Golgi residence is contained within the N-terminal part of GT, we have tried to prevent movement of Iip31 through the Golgi apparatus by exchanging N-terminal portions of Iip31 with those from GT. By constructing such hybrids, we have been able to identify domains that mediate retention of GT in the *trans* cisternae of the Golgi stack.

Results

The cytoplasmic domain of GT

cDNAs encoding Iip33 or Iip31 (see Figure 1), cloned into the expression vector pCMUIV, were expressed transiently in monolayer HeLa cells by the method of calcium phosphate transfection (see Materials and methods). Cells were fixed 72 h after transfection, processed for indirect immunofluorescence and stained using the peptide antiserum, K2, which

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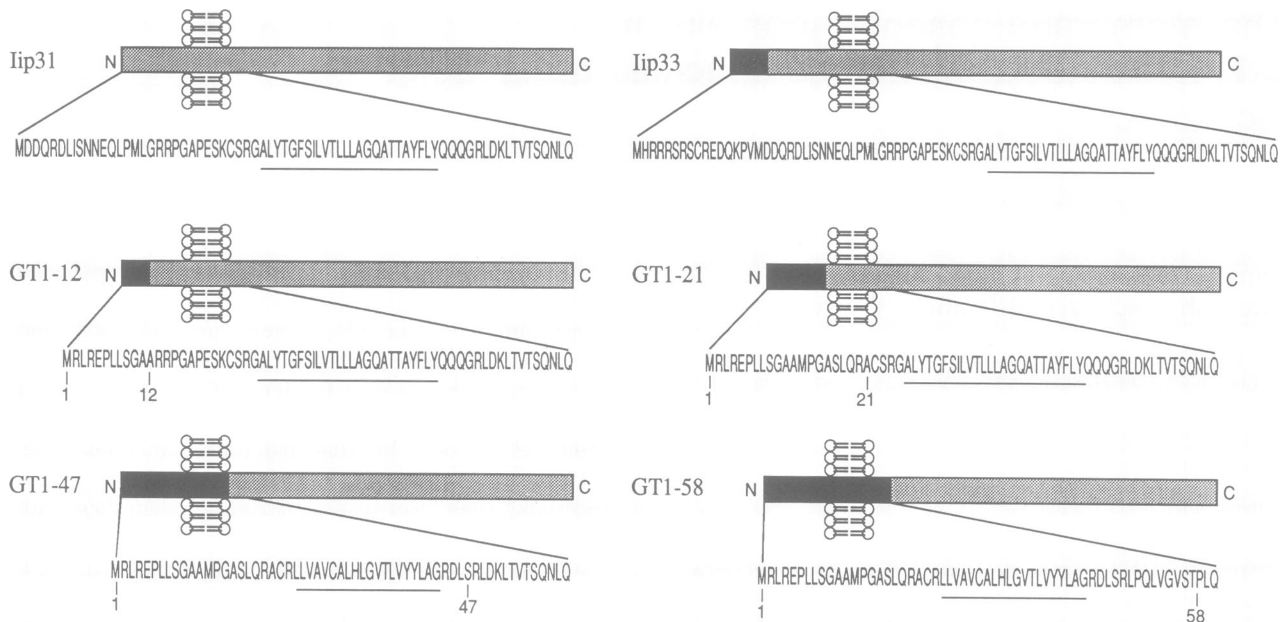

Fig. 1. Schematic representation of Iip31, Iip33 and hybrid proteins in which the cytoplasmic and membrane spanning domains have been partially or completely replaced by corresponding sequences from GT. The three domains depicted are the N-terminal (N) cytoplasmic domain, the membrane spanning domain, and the C-terminal (C) lumenal domain. The black boxes represent those parts of Iip31 which have been replaced by the corresponding sequences in GT. The expected sequence is numbered according to the published sequence for GT (Masri *et al.*, 1988). We use the term membrane spanning domain to denote those amino acids thought to interact with the fatty acid chains of the lipids (underlined) and the one or two flanking amino acids thought to interact with the lipid head groups. The hatched box in Iip33 represents the additional 16 amino acids that cause retention in the ER.

is specific for the lumenal domain of Ii. As shown in Figure 2A, cells expressing cDNA encoding Iip33 gave a staining pattern characteristic of the ER. The nuclear envelope was stained together with a reticulum which pervaded the entire cell cytoplasm. In contrast, Iip31 was localized to the cell surface and vacuolar or vesicular structures (Figure 2B) believed to represent a late compartment of the endocytic pathway (Lotteau *et al.*, 1990; Bakke and Dobberstein, 1990). The size of the vacuoles and vesicles was found to be directly proportional to the amount of DNA used for transfection.

Two chimeric cDNAs were constructed in which the region encoding the cytoplasmic domain of Ii was replaced by the corresponding portion of human GT (Masri *et al.*, 1988). Expressed hybrid proteins were expected to contain approximately half (GT1-12) or almost the entire (GT1-21) cytoplasmic domain of GT (see Figure 1). Cells expressing either one of these two hybrids showed staining of the cell surface and vacuoles and vesicles throughout the cell cytoplasm (Figure 2C and D). The staining pattern was very similar to that observed with Iip31 (Figure 2B) except that the size of the observed vacuolar and vesicular structures was consistently smaller when using the same amount of DNA. This staining pattern was rather surprising since earlier work had shown that the cytoplasmic domain of Ii was important for targeting. Since the GT cytoplasmic domain did not cause retention in the Golgi we would have expected the hybrids to move, by default, to the cell surface and not into the vacuoles and vesicles seen in Figure 2C and D.

To investigate this further, cells transfected with cDNAs encoding Iip33, Iip31, GT1-12 and GT1-21 were subjected to analysis by flow cytofluorometry (FACS). Figure 3 shows that cells expressing Iip33 had little if any of the protein on the cell surface since the distribution of fluorescence was indistinguishable from that of mock transfected cells (not shown). In cells expressing Iip31, however, almost half the cell population showed cell surface staining, in agreement with previous findings (Lotteau *et al.*, 1990; Wraight *et al.*, 1990). The proportion of cells transiently expressing Iip31 was 30–40%, as judged by indirect immunofluorescence, a less sensitive technique. This shows that all cells expressing Iip31 also expressed this protein on the cell surface. The same proportion of transiently transfected cells expressed either GT1-12 or GT1-21 but here, less cell surface expression was observed, much less than that expected if the hybrid proteins were being expressed only on the cell surface. This shows not only that the cytoplasmic domain of GT cannot retain Iip31 in the Golgi but also that this domain cannot alone be responsible for directing Iip31 into the endocytic compartment.

The cytoplasmic and membrane spanning domains of GT

To extend the portion of GT to include the region encoding the membrane spanning domain, chimeric cDNAs encoding GT1-47 and GT1-58 were constructed (see Figure 1). The latter included approximately half of a region which has been termed a stalk, linking the membrane spanning domain with the enzymic region of GT. The indirect immunofluorescence patterns, 72 h after transfection (Figure 2E and F), were found to be dramatically different from those observed for GT1-12 and GT1-21. A compact, juxtanuclear reticulum strongly reminiscent of the Golgi apparatus was seen. No significant cell surface staining was observed by FACS analysis (Figure 3) and when both hybrid proteins were over-expressed, by increasing the amount of plasmid DNA to 20 μg, neither reached the cell surface as determined by FACS analysis (data not shown). Instead, an ER staining pattern appeared in addition to the juxtanuclear Golgi (Figure

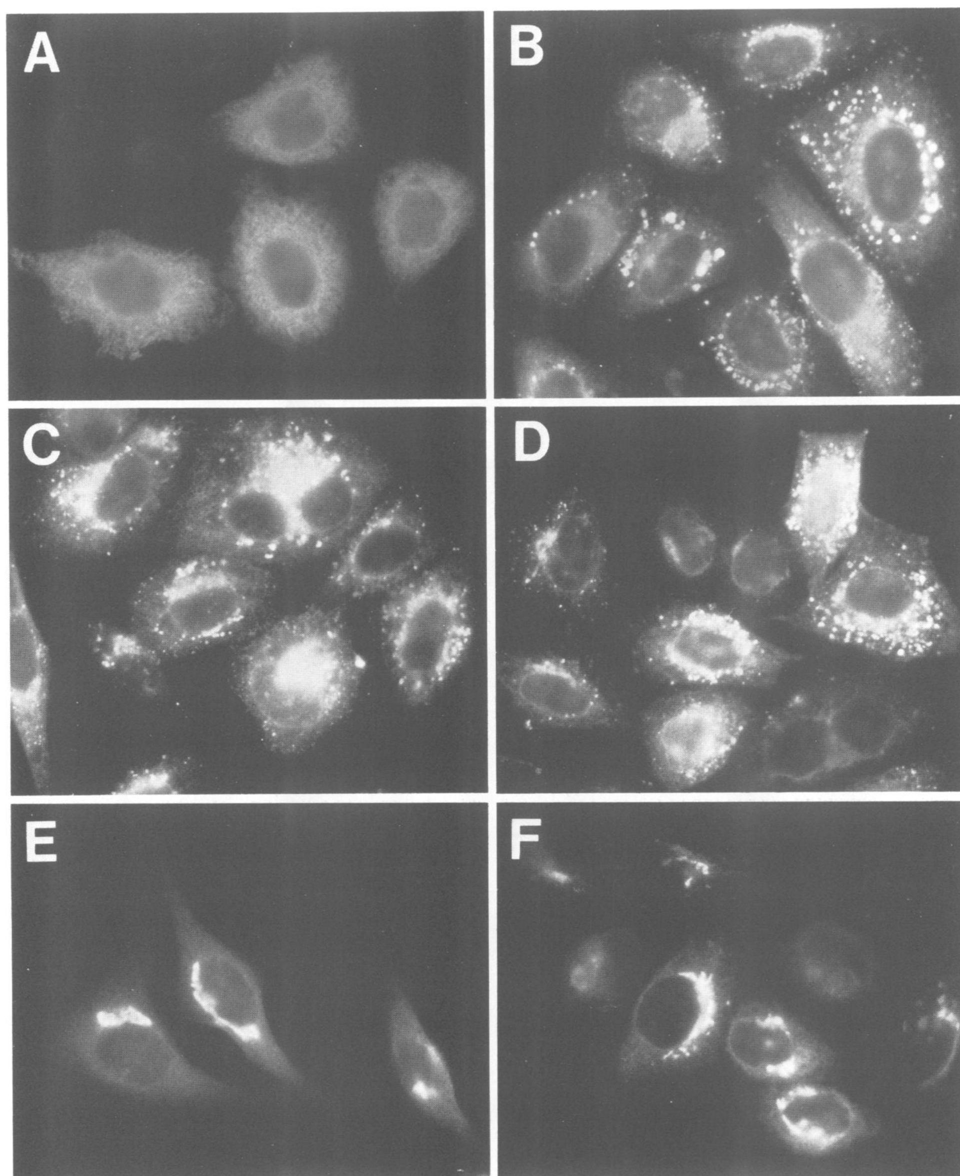

Fig. 2. Immunofluorescence microscopy of HeLa cells transfected with (**A**) Iip33, (**B**) Iip31, (**C**) GT1-12, (**D**) GT1-21, (**E**) GT1-47 and (**F**) GT1-58. Cells were fixed, permeabilized and incubated with antiserum reactive against the lumenal portion of Iip31. Bound antibody was visualized by a secondary antibody conjugated to Texas Red. Magnification at 800×.

5A) suggesting that the proteins did not pass beyond the Golgi apparatus. The fact that GT1-47 and GT1-58 were virtually indistinguishable by all criteria strongly suggests that the stalk region of GT is not required for Golgi localization.

The membrane spanning domain of GT

Since the cytoplasmic domain of GT was not sufficient to retain Iip31 in the Golgi, yet this domain together with the membrane spanning domain was, the cytoplasmic domain was truncated to determine whether the membrane spanning domain alone could cause Golgi localization. Two different chimeric cDNAs were constructed in which either half or the entire region encoding the cytoplasmic domain of GT1-47 was removed (see Figure 4). Cells expressing GT13-47, containing half the cytoplasmic domain of GT, showed a Golgi staining pattern (Figure 5B) very similar to that observed with GT1-47 (Figure 2E). A small proportion of the cells also displayed cell surface staining which was confirmed by FACS analysis using GT1-47 as a control (Figure 6A). A similar Golgi staining pattern was also observed with the second hybrid protein Δ27GT24-47 (Figure 5C) in which the entire cytoplasmic domain of GT had been removed. However, a more substantial proportion of the cells expressed the protein on the cell surface as seen by FACS analysis (Figure 6A). Treatment with cyclohex-imide for 3 h did not substantially change the staining pattern, showing that the population of hybrid protein in the Golgi was retained there and was not simply in transit to the cell surface.

The appearance of some of the hybrid proteins on the cell surface suggested that the cytoplasmic tail does play a role in retention. To determine whether the GT tail itself was needed, the cytoplasmic tail of GT1-47 was replaced with that of Iip31. Unfortunately, the resulting hybrid protein, GT24-47, failed to give rise to any positively stained cells

presumably because it could not be translocated into the ER lumen during synthesis. Instead, a 16 amino acid N-terminal portion of Iip31 was added onto Δ27GT24-47 yielding Δ15GT24-47 (see Figure 4). This hybrid protein behaved in a very similar way to GT1-47. Most of the protein product accumulated in the Golgi region (see Figure 5D) and none could be detected on the cell surface by FACS analysis (Figure 6B) indicating that complete retention had been restored.

To determine whether the entire membrane spanning domain of GT was required for Golgi retention, the cytoplasmic half of this domain was replaced in Δ15GT24-47 by the equivalent Iip31 sequence to yield Δ15GT38-47 (Figure 4). Despite having only 10 amino acids from the GT spanning domain, this hybrid protein was found mostly in the Golgi by immunofluorescence (Figure 5E) though some ER staining could also be detected. Some of this hybrid was also present on the cell surface as seen by FACS analysis (Figure 6B). Further extension of the cytoplasmic domain did not restore complete Golgi retention (data not included).

Treatment with brefeldin A

Since brefeldin A inhibits ER to Golgi transport (Fujiwara et al., 1988) by rapidly and reversibly returning Golgi residents to the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989), it is a useful tool to determine whether a protein is resident in the Golgi apparatus. Transfected cells were treated with cycloheximide for 2 h followed by a 1 h incubation with cycloheximide in the presence or absence of brefeldin A. All of the hybrid proteins that gave rise to a Golgi staining pattern in the absence of the drug were now found to display an ER staining pattern. The example shown in Figure 5F is the hybrid protein Δ15GT24-47 containing the entire GT spanning domain. The hybrid GT1-12, present on the cell surface and in vacuoles and vesicles, was also tested but did not give rise to a significant ER staining pattern upon brefeldin A treatment (data not shown).

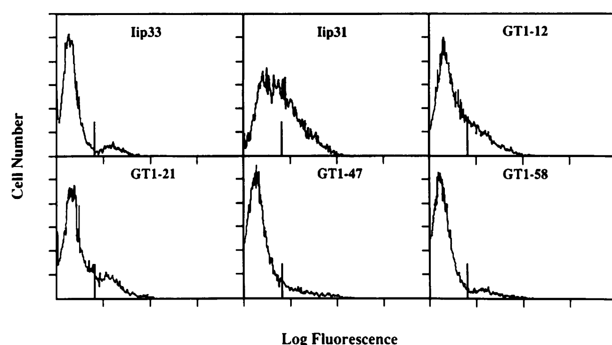

Fig. 3. FACS analysis of HeLa cells transfected with cDNAs encoding the indicated proteins and hybrids. Approximately 1×10^5 cells were analysed in each run. The proportion of cells displaying cell surface fluorescence (name of protein in parentheses) was: 10% (Iip33), 47% (Iip31), 29% (GT1-12), 25% (GT1-21), 10% (GT1-47), 11% (GT1-58) and 10% (mock transfected cells) (not shown).

Electron microscopy

The Golgi location of two of the constructs, GT1-47 and Δ15GT38-47, was confirmed by immuno-labelling of thin, frozen sections of transfected cells. In cells expressing hybrid protein containing only half of the GT spanning domain (Δ15GT38-47), label was found over an expanded compartment on one side of the Golgi stack (Figure 7C). This compartment was labelled with two or more gold particles in 19% of the nucleated cell profiles observed ($n = 63$). Note that the majority of the cells were not expressing any hybrid protein at all, in agreement with the immunofluorescence data. This expanded compartment was shown to be on the trans side of the stack by labelling with antibodies reactive against the lumenal domain of GT (Figure 7A). Such antibodies could only label endogenous GT and not the hybrid protein. Labelling was restricted, in almost all cases, to the expanded compartment. The expansion of the trans compartment proved very useful in defining the polarity of the stack and did not seem to be the result of transfection since unlabelled stacks were also expanded. Rather, the expansion of the cisternae was most likely due to fixation with formaldehyde, since this effect was absent in glutaraldehyde fixed cells.

Labelling for hybrid protein was also found over large lucent vacuoles in 8% of the cell profiles. These vacuoles were heavily labelled, some with hundreds of gold particles. While most of these cells showed small amounts of cell surface labelling, in agreement with FACS analysis, and labelling of the ER (Figure 7D), very occasionally cells showed heavy labelling of both structures. These vacuoles were often associated with cisternal-like elements (Figure 7D) and rarely with Golgi stacks though in such cases the entire stack was labelled for Ii. The vacuoles were, nevertheless, derived from the trans Golgi because most, if not all of them labelled for endogenous GT (Figure 7B). Cells expressing the hybrid protein GT1-47 gave very similar

Fig. 4. Schematic representation of the hybrid protein, GT1-47, after further truncation and substitution. Symbols as in Figure 1.

results. In 27% of the cell profiles ($n = 22$) two or more gold particles were found over an expanded *trans* cisterna (Figure 7E). A subset of the cells also showed large electron lucent vacuoles which labelled for Ii (Figure 7F) and, in most cases, for GT.

Transfected cells appeared to contain more autophago-

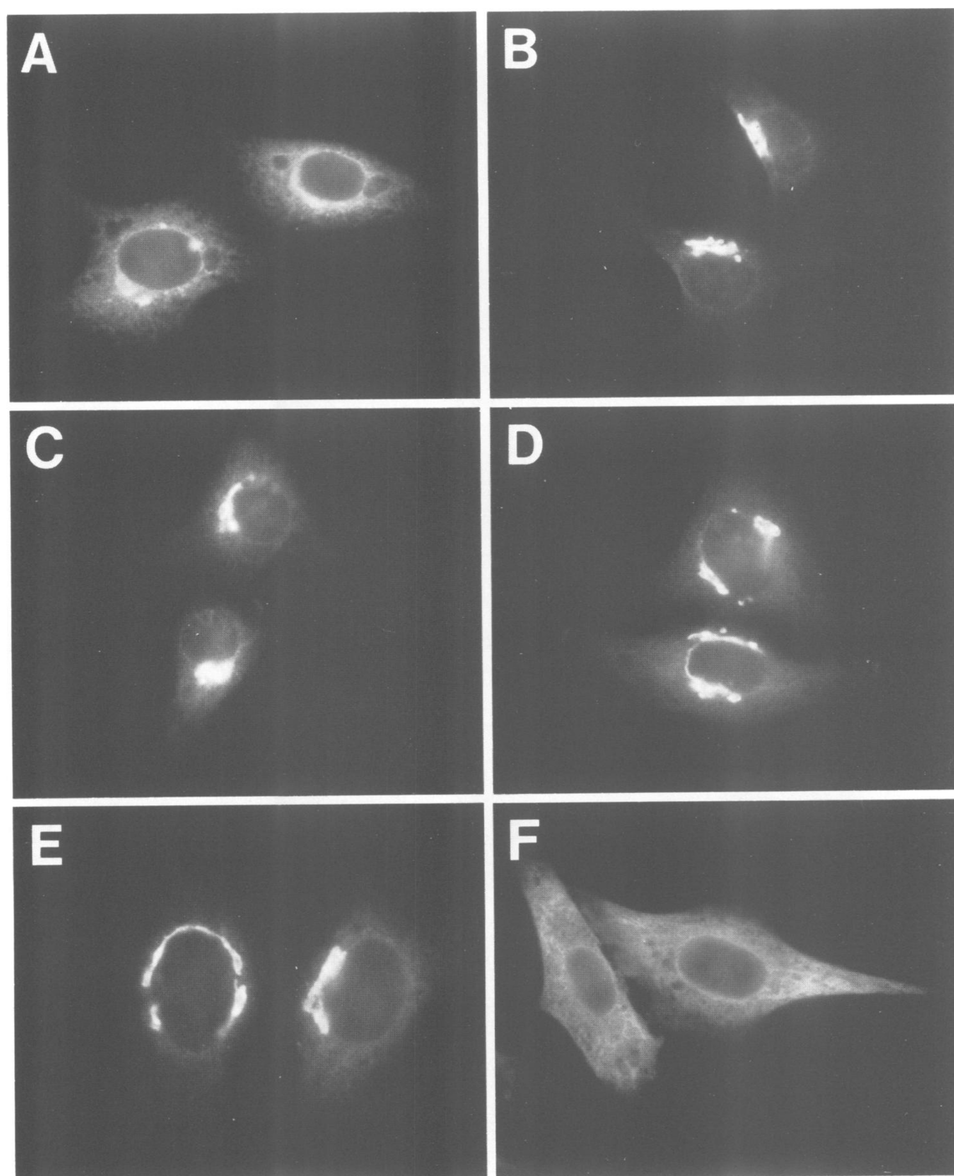

Fig. 5. Immunofluorescence of cells expressing (**A**) GT1-47, (**B**) GT13-47, (**C**) Δ27GT24-47, (**D**) Δ15GT24-47, (**E**) Δ15GT38-47 and (**F**) Δ15GT24-47. Cells were fixed, permeabilized and processed for indirect immunofluorescence using antibodies against the lumenal domain of Iip31. Note that over-expression of GT1-47 (A) and treatment with brefeldin A (F) gave rise to an ER staining pattern. Magnification at ×800.

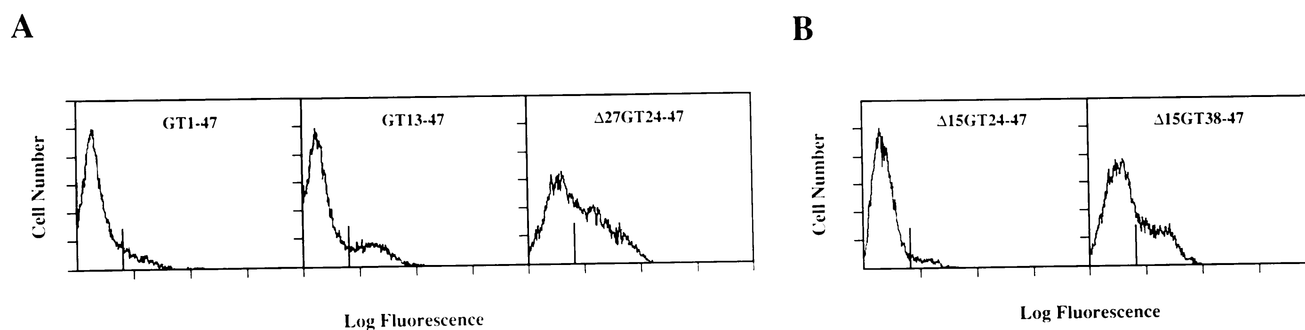

Fig. 6. FACS analysis of truncation and substitution hybrids. (**A**) Truncation of the cytoplasmic domain. The proportion of cells gated as positive for each cell population transfected with cDNA encoding GT1-47, GT13-47 and Δ27GT24-47 was 14%, 18% and 50% respectively. (**B**) Substitution within the cytoplasmic domain and part of the membrane spanning domain. Cells transfected with cDNA encoding Δ15GT24-47 and Δ15GT38-47 gave rise to cell surface expression in 7% and 32% of the analysed population, respectively.

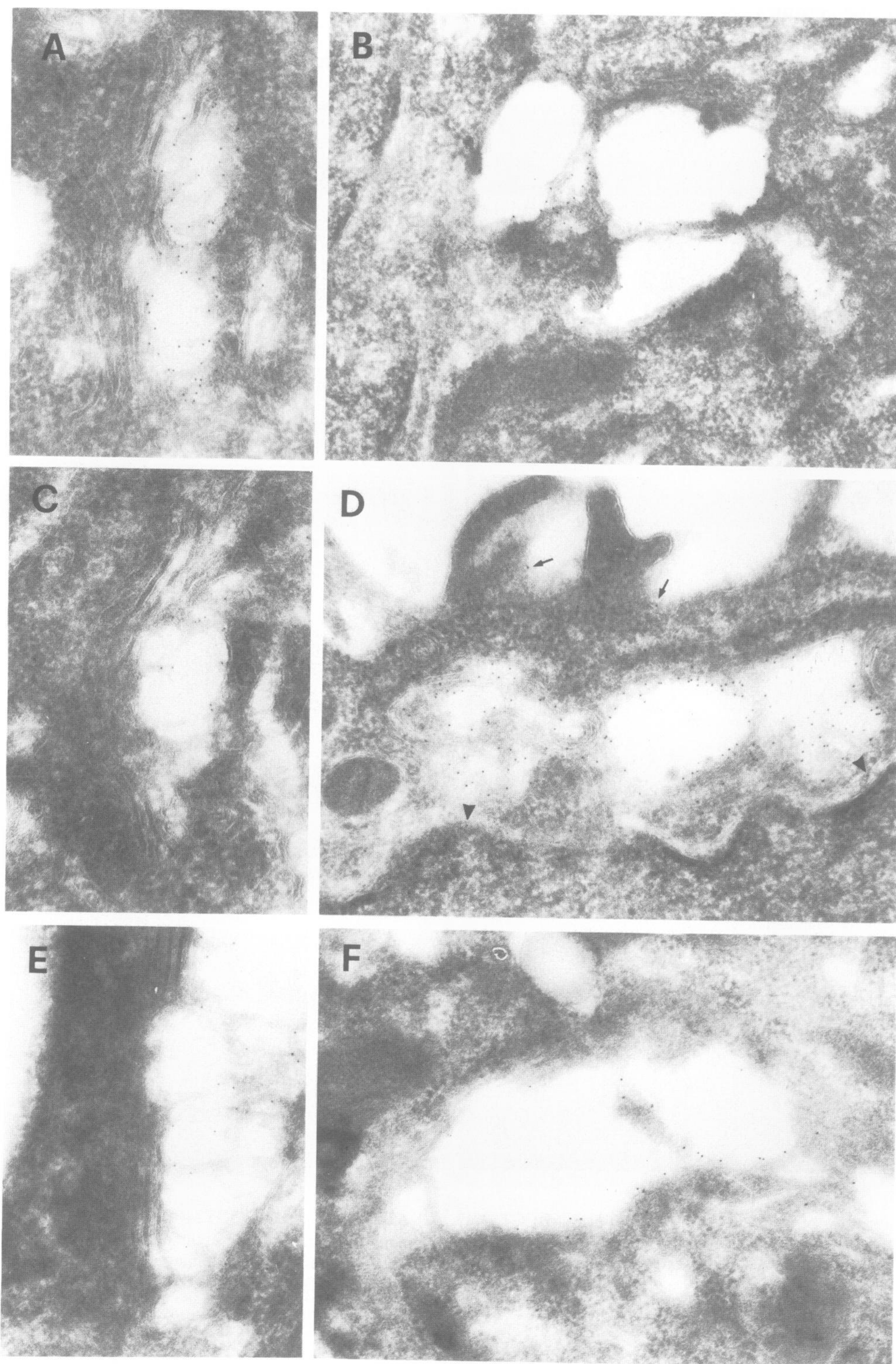

Fig. 7. Immuno-labelling for galactosyltransferase and hybrid proteins in transfected cells. HeLa cells expressing either Δ15GT38-47 (A–D) or GT1-47 (E–F), were fixed, frozen and sections labelled with antibodies either to GT (A–B) or Ii (C–F). Antibodies label the expanded compartment on the *trans* side of the Golgi stack (A, C, E) or large lucent vacuoles (B, D, F). Note the surface (arrows) and nuclear envelope (arrowheads) labelling in D. Magnifications at 52 760× (A), 39 700× (B), 40 220× (C), 48 328× (D), 56 160× (E) and 39 420× (F).

somes than normal HeLa cells, and, on occasion, very large autophagosomes were observed, some bigger than the cell nucleus. Their origin is, however, unclear because there was no apparent correlation between the presence of autophagosomes and the presence of either construct in the Golgi apparatus.

Metabolic labelling of transfected cells

Each of the hybrids was further characterized by metabolic labelling and immunoprecipitation. At 72 h after transfection, cells were labelled with [^{35}S]methionine for 15 min (see Materials and methods) followed by incubation with excess cold methionine for either 0 or 3 h. Cells were

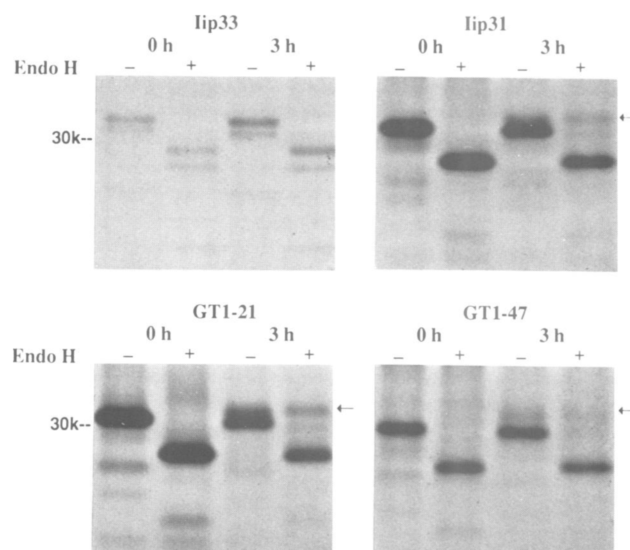

Fig. 8. Metabolic labelling of transfected cells shows partial endo H resistance of transported proteins. HeLa cells transfected with cDNAs encoding Iip33, Iip31, GT1-21 and GT1-47 were labelled, 72 h after transfection, with [^{35}S]methionine and chased in the presence of cold methionine for either 0 or 3 h. Proteins were immunoprecipitated and subjected to mock treatment (−) or deglycosylation by endo H (+) followed by SDS−PAGE analysis. Arrows point to resistant proteins. The position of a 30 kDa marker protein is indicated.

subsequently lysed and subjected to immunoprecipitation using the rabbit peptide antiserum, K2. Immunoprecipitates were next subjected to deglycosylation by endoglycosidase H (endo H). Acquisition of resistance to endo H signifies the processing of N-linked oligosaccharides by *medial* Golgi enzymes (Dunphy *et al.*, 1985). Following a 3 h chase, Iip33 did not acquire detectable endo H resistance compared with the mock treated sample (see Figure 8), consistent with Iip33 residing in the ER (Lotteau *et al.*, 1990). Iip31, however, gave rise to a partially resistant form migrating at an identical position to the somewhat slower form seen in the mock-treated sample. A very similar pattern to that of Iip31 was observed with all hybrids, and GT1-12 and GT1-47 are shown as examples. Extended pulse−chases revealed that Iip31, as well as the hybrid proteins, did not acquire complete endo H resistance under the conditions used. Instead, both sensitive and resistant forms seemed to be degraded at similar rates. The long half-life observed for Iip33 is consistent with that of a resident ER protein, whereas most of the Iip31 was found to be degraded within 4 h. Both GT1-12 and GT1-21 displayed a similar half-life to that of Iip31. The half-lives of hybrids that gave rise to a Golgi staining pattern, such as GT1-47, fell between those of Iip33 and Iip31.

Discussion

The signal for Golgi retention has been localized to the N-terminal cytoplasmic and membrane spanning domains of GT. The hybrid protein, GT1-47, containing both these domains joined to the lumenal domain of Ii, was localized to the Golgi using a number of criteria. Immunofluorescence revealed a compact juxtanuclear reticulum that strongly resembled that of the Golgi apparatus seen in HeLa cells and, at low levels of expression, this was the only organelle stained. None of the hybrid protein was present on the cell surface as shown by FACS analysis. The fluorescence pattern was not sensitive to treatment with cyclohemixide, showing that the hybrid was a resident of the Golgi, a fact confirmed by brefeldin A which caused the hybrid to redistribute to the ER. The half-life of the hybrid was intermediate between that of the ER protein, Iip33, and the endocytic protein, Iip31, consistent with a location between these two organelles. Partial resistance to endo H showed that the hybrid protein had at least reached the *medial* cisternae. Such partial resistance probably reflected the inaccessibility of the bound oligosaccharides to the processing enzymes, a feature found in other proteins (see Omary and Trowbridge, 1981; Jackson *et al.*, 1990). Finally, the precise location of the hybrid within the Golgi was determined by immuno-electron microscopy. Almost all of it was confined to the same *trans* Golgi cisternae that could be labelled using antibodies to the endogenous GT.

The retention signal appears to be in two parts with the membrane spanning domain specifying the location in *trans* cisternae. Δ15GT24-47, which has the entire spanning domain derived from GT, was localized to the Golgi by all criteria. Note that we use the term membrane spanning domain to include those amino acids that would be expected to interact with the fatty acid tails of membrane lipids and the one or two amino acids that might be expected to interact with the lipid headgroups. This construct therefore has a few amino acids on the lumenal side that could contribute to retention yet are not included in this definition of the spanning region. The next two amino acids (RL) are shared between GT and Ii. Such sites were chosen deliberately, wherever possible, to minimize the perturbation caused by putting two foreign domains together. Further work will therefore be needed to determine whether any of these lumenal amino acids play a role in the retention process.

Further truncation of this region was possible. Δ15GT38-47 had only 10 amino acids on the lumenal side of the spanning domain and this hybrid was localized to *trans* Golgi cisternae by immuno-electron microscopy. Some of this hybrid also appeared on the cell surface suggesting that this domain might be just too short for complete retention. Further work will be needed to substantiate this. In any event it is interesting that this 10 amino acid region is not homologous to the equivalent region in any of the transferases which share the *trans* cisternae or transferases present in other Golgi cisternae. The only point of note comes from comparing the membrane spanning regions of human (Grundman *et al.*, 1990) and rat (Weinstein *et al.* 1987) β-galactoside α-2,6-sialyltransferase. The lumenal half of the spanning region is, interestingly enough, the most highly conserved region. There is one other report that a membrane spanning domain is involved in retaining a protein in the Golgi. The E1 glycoprotein of infectious bronchitis virus (IBV) is a type III protein, spanning the membrane three times, and located in *cis* Golgi cisternae (Machamer *et al.*, 1990). Truncation suggests that the first spanning domain is required for retention (Machamer and Rose, 1987) though similar work on a highly related virus gave very different results (Armstrong and Patel, 1991).

The other part of the retention signal is contained within the cytoplasmic domain. Though removal of the cytoplasmic domain left intracellular staining in the Golgi region, much of the hybrid appeared on the cell surface (Δ27GT24-47). Since full retention was restored irrespective of whether the

GT or Ii cytoplasmic domain was used, this shows that this domain does not specify location but plays an accessory role in the retention process.

What might be the underlying mechanism for localizing GT to *trans* cisternae? One possibility is that the membrane spanning domain is recognized by a specific receptor. This happens during biosynthesis, so there is a precedent (Lauffer *et al.*, 1985), but one then has to explain how such a receptor, in turn, is localized to the correct cisterna(e). Another possibility is that GT is retrieved by a mechanism analogous to that suggested for soluble ER proteins terminating in -KDEL (see Warren, 1987; Pelham, 1989). This could help explain the retrograde pathway from the Golgi revealed by brefeldin A (Lippincott-Schwartz *et al.*, 1989) but would require a recycling receptor that recognizes the membrane spanning domain of a protein. There is, as yet, no example of such a receptor. Another possibility is that GT oligomerizes upon recognition of identical or related molecules in the *trans* cisternae, forming a complex of sufficient size to prevent it entering Golgi transport vesicles. Such 'kin recognition' could be mediated through the membrane spanning domain as exemplified by several studies. Bormann *et al.* (1989) have demonstrated preferential association between a synthetic peptide corresponding to the membrane spanning domain of glycophorin A and native glycophorin. The membrane spanning domain of the α chain of the T-cell receptor has charged residues that have recently been shown to mediate dimerization with the CD3 δ chain (Cosson *et al.*, 1991). Lastly, we have been able to construct a mini-protein comprising the first 80 amino acids of Iip31 and have found that this can form stable oligomers (T.Nilsson and D.Mackay, unpublished data) though it remains to be seen if this is also true for GT and other Golgi residents. The spanning domain alone would not be expected to form extensive oligomers and this might be the function of the accessory, cytoplasmic domain. If the spanning domain generates dimers then the cytoplasmic domain could interact, directly or indirectly, with neighbouring dimers so that extensive oligomers could form. Alternatively, the cytoplasmic domain might stabilize the dimer so that it could interact with neighbouring dimers through the lumenal domain. An attractive feature of oligomerization is that it will be dependent on the concentration of residents of a particular cisterna(e). Over-expression would lead to premature oligomerization which would explain why the Golgi hybrids back up into the ER and do not move past the Golgi.

An oligomerization mechanism has general appeal. Earlier work had shown that truncation of Ii caused the protein to appear on the cell surface arguing that the information for sorting to the endocytic pathway was contained in the cytoplasmic domain (Lotteau *et al.*, 1990; Bakke and Dobberstein, 1990). We have now shown that this domain can be replaced by that from GT without affecting the final destination, suggesting that the information resides elsewhere, perhaps in the membrane spanning domain. This is supported by the fact that replacing this domain alone in Ii by the equivalent GT sequence results in Golgi retention. Hybrid proteins in which the spanning domain of GT is replaced by that from Ii will be required to confirm this suggestion but, if true, then the same type of signal would specify the location of proteins in the Golgi, along the endocytic pathway, and perhaps elsewhere.

Materials and methods

Recombinant DNAs

cDNAs encoding Iip33 and Iip31 inserted into the eukaryotic expression vector pCMUIV have been described previously (Lotteau *et al.*, 1990). Each chimeric cDNA was constructed by means of direct cloning of synthetic oligonucleotides. To generate GT1-12 and GT1-21, complementary oligonucleotides encoding amino acids 1–12 or 1–21 (Masri *et al.*, 1988) were cloned into Iip31Δ16 between the *Xba*I and *Sal*I or *Xba*I and *Sac*II sites, respectively (see Lotteau *et al.*, 1990). In order to generate further chimeras, partially overlapping synthetic oligonucleotides encoding the entire cytoplasmic and membrane spanning domains of either Iip31 (Strubin *et al.*, 1984) or GT (Masri *et al.*, 1988) were cloned into pBS between *Xba*I and *Pst*I. After sequencing, inserts were excised with *Xba*I and *Pst*I and cloned into Iip31Δ16 between the *Xba*I and *Pst*I sites for expression. Each construct was tested for expression by transient transfection followed by immunoprecipitation and SDS–PAGE analysis. The apparent molecular weight of each hybrid protein was found to be in agreement with the expected one.

Cell culture and transfection

Monolayer HeLa cells (ATCC CCL 185) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Northumbria Biologicals Ltd) supplemented with 10% fetal calf serum, penicillin and streptomycin at 100 μg/ml. Transfection was carried out essentially as described previously (Nilsson *et al.*, 1989) with the following modifications. Routinely, each transfection was carried out using 5 μg plasmid DNA mixed with 20 μg carrier DNA (pCMUIV lacking cDNA). For over-expression, these amounts were reversed. Similarly, to decrease the expression level, the amount of expression vector containing cDNA was kept at 0.5 μg and carrier DNA at 24.5 μg.

Immunofluorescence and cytofluorometric analysis

Transfected cells were seeded out onto coverslips 48 h after transfection. The following day, cells were fixed and processed for indirect immunofluorescence essentially as described by Louvard *et al.* (1982). Briefly, cells were washed extensively in PBS followed by fixation in 3% formaldehyde–PBS. Cells were permeabilized by incubation in 0.1% Triton X-100–PBS for 5 min followed by incubation in 0.2% fish skin gelatin–PBS (FPBS). Coverslips were inverted over 100 μl of the appropriate primary antibody and incubated for 20 min followed by extensive washing with FPBS. Secondary antibodies conjugated to Texas Red (Vector Laboratories) or FITC (Dakopatts) were used to visualize the primary antibody. Fluorescence microscopy was performed using a Zeiss Axiophot microscope. Images were recorded on Ilford HP5 Plus film.

Cytofluorometry was carried out as described by Nilsson *et al.* (1989) with the following modifications. Cells were removed from the Petri dish by incubation in PBS–EDTA for 5 min at 37°C. The amount of bound primary antibody (K2) was quantified indirectly using FITC-conjugated, goat anti-rabbit IgG (Dakopatts). Cells were fixed in 3% formaldehyde before being subjected to analysis using a FACScan (Becton Dickinson Immunocytometry Systems).

Metabolic labelling, antibodies, immunoprecipitation, SDS–PAGE and endo H treatment

Radiolabelling and immunoprecipitation were carried out as described previously (Nilsson *et al.*, 1989). Samples were precipitated using the peptide antiserum, K2, specific for the Iip31 lumenal domain (V.Quaranta, unpublished result). Antibodies used in immunoelectron microscopy were rabbit polyclonal antisera raised against the lumenal domain of Ii (Lipp and Dobberstein, 1986) and rabbit polyclonal antisera that recognized only the deglycosylated lumenal domain of GT (Lucocq *et al.*, 1987). SDS–PAGE was performed essentially as described by Maizel (1969) and endo H treatment as described by Sege *et al.* (1981).

Electron microscopy

Monolayer HeLa cells were fixed with 8% paraformaldehyde in 0.25 M HEPES pH 7.2 for at least two days. Cells were subsequently washed in HEPES buffer, released by scraping using a rubber policeman, pelleted and incubated in 2.3 M sucrose–PBS on ice before freezing in liquid nitrogen. Ultrathin frozen sections were prepared at –100°C using glass knives and picked up on droplets of 2.1 M sucrose in PBS containing 0.5% (w/v) fish skin gelatin. Sections were next incubated at room temperature in 0.1 M ammonium chloride in PBS for 5 min followed by a 5 min incubation in PBS containing fish skin gelatin. Sections were then incubated with the

appropriate antibody for 20 min and washed in PBS for 15 min before incubation with protein A−gold (6.2 nm). Following several washes in PBS and distilled water, sections were embedded and contrasted in methylcellulose−uranyl acetate as described by Griffiths *et al.* (1984). Observations were carried out using a Philips 300 transmission electron microscope at 60 or 80 kV.

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