Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins

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Polarized expression of most epithelial plasma membrane proteins is achieved by selective transport from the Golgi apparatus or from endosomes to a specific cell surface domain. In Madin–Darby canine kidney (MDCK) cells, basolateral sorting generally depends on distinct cytoplasmic targeting determinants. Inactivation of these signals often resulted in apical expression, suggesting that apical transport of transmembrane proteins occurs either by default or is mediated by widely distributed characteristics of membrane glycoproteins. We tested the hypothesis of *N*-linked carbohydrates acting as apical targeting signals using three different membrane proteins. The first two are normally not glycosylated and the third one is a glycoprotein. In all three cases, *N*-linked carbohydrates were clearly able to mediate apical targeting and transport. Cell surface transport of proteins containing cytoplasmic basolateral targeting determinants was not significantly affected by *N*-linked sugars. In the absence of glycosylation and a basolateral sorting signal, the reporter proteins accumulated in the Golgi complex of MDCK as well as CHO cells, indicating that efficient transport from the Golgi apparatus to the cell surface is signal-mediated in polarized and non-polarized cells.

Keywords: cell polarity/glycosylation/Golgi apparatus/membrane protein sorting

Introduction

Polarized epithelial cells possess two functionally and compositionally distinct cell surface domains that are separated from each other by tight junctions. The biogenesis and maintenance of these two cell surface domains require the continuous sorting of newly synthesized and internalized plasma membrane proteins (Wandinger-Ness and Simons, 1990; Rodriguez-Boulant and Powell, 1992; Matter and Mellman, 1994). After synthesis in the endoplasmic reticulum, apical and basolateral plasma membrane proteins are transported together to and through the Golgi apparatus. The *trans*-Golgi network (TGN) is then the first major sorting site for specific apical and basolateral transport. Basolateral targeting in the TGN is very efficient but can involve transient appearance in endosomes (Futter *et al.*, 1995; Leitinger *et al.*, 1995). The efficiency of

apical targeting in the TGN depends on the epithelial cell type as well as on the protein analyzed and, therefore, can occur by a direct pathway or by an indirect route via the basolateral cell surface (Bartles *et al.*, 1987; Le Bivic *et al.*, 1990; Matter *et al.*, 1990; Bonilha *et al.*, 1997). Distinct pathways from the Golgi apparatus to the cell surface are not specific for epithelial cells but can also be observed in fibroblasts, suggesting that similar mechanisms for cell surface transport might be used by polarized and non-polarized cells (Musch *et al.*, 1996; Yoshimori *et al.*, 1996).

Basolateral sorting of several membrane proteins has been shown to depend on distinct cytoplasmic targeting determinants that, in some cases, depend on a tyrosine motif or a double leucine motif (Matter and Mellman, 1994; Keller and Simons, 1997). Basolateral targeting determinants can be divided into those that are co-linear with a clathrin-coated pit domain and those that are not. It is not known whether these different types of basolateral targeting determinants are recognized by the same sorting machinery.

Inactivation of basolateral sorting determinants often led to apical expression, suggesting that apical sorting either occurs by default or is mediated by widely distributed common features of membrane glycoproteins. Apical sorting has been attributed to the formation of glycolipid- and cholesterol-containing membrane subdomains (i.e. rafts) (Rodriguez-Boulant and Powell, 1992; Simons and Ikonen, 1997). Apical membrane proteins were proposed to interact with these rafts in the TGN due to special characteristics of their membrane anchors [e.g. glycosylphosphatidylinositol (GPI)-anchored proteins, influenza virus neuraminidase and hemagglutinin (HA): Kundu *et al.*, 1996; Keller and Simons, 1997; Scheiffele *et al.*, 1997]. In some epithelial cell types, apical expression of membrane proteins is independent of glycolipid and GPI-anchored protein sorting, indicating that the sorting mechanisms for these membrane components are not necessarily related (Zurzolo *et al.*, 1994; Weimbs *et al.*, 1997). Apical secretion of some secretory proteins appears to be mediated by *N*-linked oligosaccharides but can also occur independently of *N*-linked glycans (Scheiffele *et al.*, 1995; Marzolo *et al.*, 1997). It is not known whether *N*-linked carbohydrates can mediate apical targeting of membrane proteins and whether efficient cell surface transport occurs in the absence of a targeting determinant (Weimbs *et al.*, 1997).

Here, we tested the hypothesis of *N*-linked oligosaccharides as apical sorting signals for membrane proteins by analyzing three different model proteins. Apical sorting of all three membrane proteins was found to depend on the presence of *N*-linked glycans. In the presence of a dominant basolateral sorting signal, the presence or absence of sugars did not affect cell surface transport. In the absence of apically sorting oligosaccharides and

basolaterally sorting cytoplasmic domain determinants, the proteins accumulated in the Golgi complex in polarized Madin–Darby canine kidney (MDCK) cells as well as in non-polarized Chinese hamster ovary (CHO) cells, indicating that efficient transport from the Golgi complex to the cell surface does not occur by default. Thus, *N*-linked oligosaccharides can act as determinants for cell surface transport of membrane proteins in polarized and non-polarized cells and specify apical expression in epithelial cells.

Results

A large fraction of C-terminally truncated occludin accumulates in the Golgi complex

The tight junction protein occludin is a polytopic membrane protein with both termini in the cytosol (Furuse *et al.*, 1993; for review, see Matter and Balda, 1998b). Several observations suggest that biosynthetic transport of occludin to tight junctions involves the basolateral membrane: small amounts of endogenous occludin can be detected over the entire basolateral membrane (Sakakibara *et al.*, 1997); mutations affecting the integration of occludin into tight junctions result in basolateral accumulation of the protein after direct basolateral transport (Matter and Balda, 1998a); and the C-terminal cytoplasmic domain of occludin is sufficient to mediate efficient basolateral transport of a reporter protein, indicating that it contains a basolateral targeting determinant (Matter and Balda, 1998a).

We recently generated stable MDCK cell lines constitutively expressing wild-type or C-terminally truncated mutants of occludin (Balda *et al.*, 1996a). Most of the synthesized wild-type occludin was transported to the cell surface where it became integrated into tight junctions and only a small amount remained intracellularly. Of a truncated protein (HAoccludinCT3) that lacked the entire C-terminal cytoplasmic domain, which mediates basolateral targeting, only a fraction was transported to tight junctions and the remainder accumulated in an intracellular compartment reminiscent of the Golgi complex (Balda *et al.*, 1996a; Matter and Balda, 1998a). C-terminally truncated occludin was found to be still capable of oligomerizing with endogenous occludin, indicating that the deletion did not cause a general conformational defect and suggesting that the fraction transported to tight junctions was dragged to the cell surface by endogenous occludin (Chen *et al.*, 1997; Matter and Balda, 1998a). To test the nature of this intracellular compartment, we processed MDCK cells expressing HAoccludinCT3 or HAoccludin for immunofluorescence using antibodies specific for the epitope-tagged occludin and for giantin, a resident protein of the Golgi complex (Linstedt and Hauri, 1993). The samples were then analyzed by confocal microscopy.

In filter-grown, fully polarized MDCK cells, a large fraction of HAoccludinCT3 accumulated intracellularly (Figure 1A1), and this intracellular staining largely overlapped with the fluorescence obtained with anti-giantin antibodies (Figure 1B1). In contrast, only a small amount of HAoccludin could be detected at the level of the Golgi complex (Figure 1B1, anti-HA; B2, anti-giantin). Since the section was not taken at the level of the tight junctions

and the samples were fixed with a method that is optimal for the intracellular staining but results in poor junctional labeling, only a little fluorescence can be seen. In immunoblots, these cell lines were found to express similar amounts of protein (Figure 2A). To confirm the colocalization of HAoccludinCT3 and giantin, we repeated the same experiment with low confluent MDCK cells grown on coverslips; this culture condition results in flatter cells and, therefore, it is easier to perform co-localization experiments. Figure 1C shows that intracellular HAoccludinCT3 co-localized with giantin also under these conditions. HAoccludin also exhibited some intracellular staining, but in a more punctate manner and with little overlap with the Golgi marker (Figure 1D1, anti-HA; D2, anti-giantin). If HAoccludinCT3-expressing cells were labeled with anti-HA antibody and protein A–gold and examined by electron microscopy, the labeling was also detected over the Golgi complex (Figure 1E). The labeling appeared to be distributed over the entire Golgi apparatus and did not seem to be restricted to a particular subcompartment. Thus, removal of the C-terminal cytoplasmic domain resulted in accumulation of large amounts of occludin in the Golgi complex. Since the deleted C-terminal domain contains a basolateral sorting signal (Matter and Balda, 1998a), occludin is the first case where removal of a basolateral targeting determinant did not result in efficient apical transport but rather in accumulation in the Golgi complex.

N-linked carbohydrates mediate cell surface transport of truncated occludin

Because occludin is not a glycoprotein, it is conceivable that the absence of sugars is the reason for the accumulation of truncated occludin in the Golgi complex. *N*-linked glycosylation was shown to be involved in apical targeting of some soluble proteins and in cell surface transport of a chimeric membrane protein in fibroblasts (Guan *et al.*, 1985; Scheiffele *et al.*, 1995). Therefore, we introduced *N*-linked glycosylation sites into both extracellular loops of HAoccludinCT3, resulting in HAoccludinCT3GL1+2. The immunoblot in Figure 2A shows that the stably expressed protein appeared as two bands, both with a higher molecular weight than the protein with no glycosylation sites. The two bands appear to represent the high-mannose and the complex glycosylated forms since there was a precursor–product relationship between them in pulse–chase experiments (not shown). If the cells were incubated with tunicamycin, an inhibitor of *N*-linked glycosylation, the protein had the same molecular weight as HAoccludinCT3, indicating that the mutant protein was indeed glycosylated.

Serial confocal sectioning showed that HAoccludinCT3GL1+2 was expressed mainly at the apical cell surface (Figure 1F1, apical; F1', lateral). Double immunofluorescence with giantin showed that there was no visible accumulation of the transfected protein in the Golgi complex (Figure 1F1', anti-HA; F2', anti-giantin). When the cells were treated for 24 h with tunicamycin, however, almost all of the transfected protein was no longer glycosylated (Figure 2A) and the protein started to accumulate in the Golgi complex and in junctions (Figure 1G1, anti-HA; G2, anti-giantin). In cells expressing full-length occludin with glycosylation sites, only a small amount of

Fig. 1. Carbohydrate-dependent apical targeting of truncated occludin in MDCK cells. Confocal sections of MDCK cells expressing HAoccludinCT3 (A and C) or HAoccludin (B and D) grown on filters (A and B) or on coverslips (C and D) were processed for immunofluorescence using the polyclonal anti-HA antibody, to visualize transfected occludin (A1, B1, C1 and D1) and a monoclonal antibody specific for the Golgi protein giantin (A2, B2, C2 and D2). The dotted linear staining between the cells reflects HAoccludinCT3 integrated into tight junctions (bar: A and B, 3 μ m; C and D, 5 μ m). (E) Electron micrograph of filter-grown MDCK cells expressing HAoccludinCT3 that were labeled prior to embedding in Epon using the monoclonal anti-HA antibody and protein A-gold (bar, 100 nm). (F) Confocal sections derived from the apical (F1 and F2) and middle (F1' and F2') part of a monolayer of cells stained for HAoccludinCT3GL1+2 (F1 and F1') and giantin (F2 and F2') (bar, 6 μ m). MDCK cells expressing HAoccludinCT3GL1+2 (G) or HAoccludinGL1+2 (H) were treated with 12 μ g/ml tunicamycin for 24 h and then stained with the anti-HA (G1 and H1) and the anti-giantin (G2 and H2) antibodies. The tunicamycin treatment resulted in some swelling of the Golgi apparatus that was independent of the transfected construct (bar, 3 μ m).

protein was detected at the level of the Golgi complex after incubation with tunicamycin (Figure 1H1, anti-HA; H2, anti-giantin), even though it was also no longer glycosylated (Figure 2A). Apical targeting of truncated occludin was also observed with constructs containing only one of the two glycosylation sites (not shown). Thus, carbohydrates are only important for cell surface transport of the apically expressed mutant but not of the full-length protein.

To test whether apical expression was due to direct apical transport of newly synthesized protein, we combined metabolic labeling and cell surface biotinylation. Since occludin does not have extracellular primary amino groups,

we used biotin-hydrazide to biotinylate the carbohydrates (Braendli *et al.*, 1990). Figure 2B shows that HAoccludinCT3GL1+2 was transported directly to the apical cell surface. Because glycosylated full-length occludin is basolaterally transported (Matter and Balda, 1998a), the basolateral sorting signal in the C-terminal domain of occludin is dominant over the apically sorting carbohydrates.

Since non-glycosylated mutant occludin might be retained in the Golgi complex by interactions with other junctional components, we transfected these mutants also into CHO fibroblasts, which do not have tight junctions. As in MDCK cells, non-glycosylated truncated occludin

Fig. 2. Expression and glycosylation of transfected proteins. (A) Immunoblots with the anti-HA monoclonal antibody of total extracts of cells expressing truncated occludin with (HoccCT3GL1+2) or without (HoccCT3) glycosylation sites or full-length occludin with (HoccGL1+2) or without (Hocc) glycosylation sites (-T, untreated; +T, cells were incubated with tunicamycin for 24 h). (B) The cell surface appearance of newly synthesized HAoccludinCT3GL1+2 was assayed with biotin-hydrazide after metabolic labeling and different times of chase (A, apical; B, basolateral). (C) Immunoblots of immunoprecipitated glycosylated (MECAG) or non-glycosylated (MECA) chimeric ERGIC-53 (-T, untreated; +T, cells were incubated with tunicamycin for 16 h). The lowest band in all lanes represents the antibody heavy chain. (D) MDCK cells expressing Fc-LDL receptor chimeras were incubated for 16 h without (-T) or with (+T) tunicamycin. The cells were then lysed in SDS-PAGE sample buffer, fractionated on 8–20% gradient gels and transferred to nitrocellulose. The membranes were then probed for the chimera with a polyclonal anti-Fc receptor antibody.

Fig. 3. Accumulation of HAoccludinCT3 (A), HAoccludin (B) or HAoccludinCT3GL1+2 (C) in the Golgi complex of CHO cells shown with anti-HA (A1, B1 and C1) and anti-giantin (A2, B2 and C2) antibodies. Shown are confocal sections through the middle of the cells containing the Golgi complex (bar, 4 μ m).

localized mainly in the Golgi complex (Figure 3A1, anti-HA; A2, anti-giantin). In contrast, only a small amount of full-length HAoccludin (Figure 3B1, anti-HA; B2, anti-giantin) and of the glycosylated deletion mutant (Figure 3C1, anti-HA; C2, anti-giantin) stayed in the Golgi complex. Thus, *N*-linked carbohydrates can mediate exit from the Golgi and cell surface transport of mutant occludin in both epithelial cells and fibroblasts.

Glycosylation-dependent cell surface transport of ERGIC-53 chimeras

Since the above-described observations could be due to very specific characteristics of occludin, we next tested ERGIC-53, a non-glycosylated protein that recycles between the endoplasmic reticulum and the *cis*-Golgi (Schweizer *et al.*, 1988). To study cell surface transport of this protein, it was necessary to remove the parts of the protein that mediate retention in the early secretory pathway (Itin *et al.*, 1995; Kappeler *et al.*, 1997). Therefore, we linked the ectodomain of ERGIC-53 containing a myc epitope to the transmembrane domain of CD4 and a polyalanine cytoplasmic tail. This chimeric protein, together with a version of it with two glycosylation sites and myc-tagged wild-type ERGIC-53 were then stably expressed in MDCK cells. Glycosylation of ERGIC-53 at the sites used here did not affect oligomerization of the chimera into dimers and hexamers (not shown). The immunoblots in Figure 2C show that glycosylated chimeric ERGIC-53 (MECAG) had a higher molecular weight than the non-glycosylated version (MECA) and that this difference disappeared when the cells were treated with tunicamycin (the lower molecular weight band in all lanes represents the antibody heavy chain since immunoprecipitates were used for the immunoblots). Thus, both proteins could be expressed and sugars were linked to the chimera with glycosylation sites.

The subcellular distribution was then analyzed by immunofluorescence combined with confocal microscopy. Analysis of low confluence cells grown on coverslips showed a slightly diffuse perinuclear staining for myc-tagged ERGIC-53 typical for the intermediate compartment (Figure 4A). In contrast, the non-glycosylated chimera gave a very distinct and concentrated perinuclear fluorescence indicative of the Golgi complex and only a little cell surface fluorescence (Figure 4B). Strikingly, the glycosylated variant was expressed at the cell surface (Figure 4C). Thus, *N*-linked carbohydrates are also able to mediate cell surface transport of the ERGIC-53 chimera. In contrast to what was observed with the mutant occludin, there was still a fraction of glycosylated chimera in the Golgi. Presently, it is not clear whether this incomplete effect was due to differences in the processing of the carbohydrates (i.e. a different composition of the oligosaccharides), the distance of the carbohydrates from the membrane (compared with the extended structure of ERGIC-53, the extracellular loops of occludin are fairly small) or to a lower general transport competence of ERGIC-53.

While we observed here only a small amount of cell surface expression of the non-glycosylated ERGIC-53 chimeras, a fraction of wild-type and non-glycosylated mutant ERGIC-53 had been shown previously to be able to reach the cell surface in transiently transfected Cos cells (Kappeler *et al.*, 1994). While the transport efficiencies of identical constructs with or without glycosylation sites were not determined in these experiments, the higher expression levels in the Cos system may have aided the detection of cell surface fluorescence due to inefficient cell surface transport. Additionally, we observed that the transport phenotypes were stronger the longer the cells were cultured before the experiment, suggesting that the

Fig. 4. N-linked glycan-induced apical expression of chimeric ERGIC-53. Subconfluent cells expressing wild-type (A), chimeric (B) or glycosylated chimeric ERGIC-53 (C) were stained with the anti-ERGIC-53 monoclonal antibody (bar, 6 µm). (D) Cell surface staining of filter-grown non-permeabilized MDCK cells expressing the glycosylated chimera (D1 and D2, apical; D3, middle; D4, basal) (bar, 10 µm). In cells expressing the non-glycosylated chimera, no surface staining could be observed (not shown).

state of differentiation influences the rates of cell surface transport.

Since biochemical assays to determine cell surface polarity of the ERGIC-53 chimera were not successful, we bound the antibody against ERGIC-53 from both the apical and the basolateral sides to non-permeabilized MDCK cells grown on filters and analyzed the distribution by confocal microscopy. This experimental protocol results in efficient staining of both apically and basolaterally expressed proteins (Matter and Balda, 1998a). The serial confocal sections in Figure 4D show that this technique resulted in strong staining of the apical membrane and only faint labeling of the basolateral membrane. The same cell surface distribution was observed when permeabilized cells were labeled (not shown), indicating that the preferential apical staining was not due to differential accessibility of the antibody to the apical and basolateral cell surface domains. Glycosylation-dependent cell surface transport and a preferential apical distribution were also obtained when the lectin domain in ERGIC-53 was inactivated by substituting Asn156 by alanine (Itin *et al.*, 1996), excluding the possibility that the sugar-binding activity of the ERGIC-53 ectodomain caused the transport phenotypes described here (not shown). Moreover, no cell surface fluorescence was detected in cells expressing the non-glycosylated chimera or the chimera with glycosylation sites in the presence of tunicamycin (not shown). Thus, carbohydrates are also able to mediate apical expression of the ERGIC-53 chimera.

Efficient cell surface transport of chimeric Fc receptors can be mediated by either N-linked glycans or the cytoplasmic domain of the LDL receptor

We next tested the importance of sugars for apical transport with a protein that is normally a glycoprotein. We took previously described chimeras consisting of the ecto- and transmembrane domain of the mouse Fc receptor for IgG and different parts of the cytoplasmic domain of the human low density lipoprotein (LDL) receptor (FcLR), which contains two basolateral targeting determinants

(Matter *et al.*, 1992). FcLR5–22 is a predominantly apically expressed chimera that contains only the clathrin-coated pit domain of the LDL receptor while FcLR5–50 contains the entire cytoplasmic domain and is basolaterally expressed. These constructs allowed us to test the importance of sugars for apical and basolateral transport with almost identical glycoproteins. Additionally, such chimeric Fc receptors, in this context, can be transported along all relevant biosynthetic and endocytic transport routes (Matter *et al.*, 1993).

To test the importance of sugars, we incubated stably transfected cells with tunicamycin for 14 h. This resulted in a shift to a lower molecular weight and a sharp band in immunoblots with anti-Fc receptor antibodies, indicating that there was essentially no glycosylated receptor left in tunicamycin-treated cells (Figure 2D). The subcellular distribution was then determined by immunofluorescence. Confocal sections showed that the predominantly apically expressed chimera FcLR5–22 accumulated intracellularly in the presence of tunicamycin (Figure 5A, control; B, tunicamycin). The absence of apical as well as lateral staining suggests that significant amounts of FcLR5–22 were not expressed at the cell surface of tunicamycin-treated cells. This was confirmed by the absence of a significant fluorescence signal when filter-grown tunicamycin-treated cells were incubated with anti-Fc receptor antibodies on ice without permeabilization (not shown) or by the inefficient labeling when the same experiment was repeated with subconfluent cells but performing the antibody incubation at 37°C in order to see also chimera only transiently expressed at the cell surface (Figure 5G, control; H, tunicamycin). Double immunofluorescence with anti-giantin antibody showed that the intracellular staining for FcLR5–22 largely overlapped with the Golgi apparatus (Figure 5E1, anti-Fc receptor; E2, anti-giantin). These observations indicate that N-linked sugars are also important for efficient exit of FcLR5–22 from the Golgi complex and its cell surface expression.

We next tested the importance of N-linked glycans for cell surface transport of FcLR5–50, the chimera containing

Fig. 5. Effect of tunicamycin on the subcellular distribution of chimeric Fc receptors in MDCK cells. Filter-grown MDCK cells expressing the apically expressed chimera FcLR5-22 (A, control; B, incubated for 16 h with tunicamycin) or the basolaterally expressed receptor FcLR5-50 (C, control; D, incubated for 16 h with tunicamycin) were stained with the anti-Fc receptor antibody. Shown are sections from the apical (1), middle (2) and basal (3) region of the monolayer (bar, 10 μ m). (E and F) Co-staining with anti-giantin antibody showed that tunicamycin induced co-localization of the 'apical' chimera FcLR5-22 (E1, anti-Fc receptor; E2, anti-giantin) but not of FcLR5-50 (F1, anti-Fc receptor; F2, anti-giantin) (bar, 6 μ m). Subconfluent cells expressing FcLR5-22 (G, control; H, pre-incubated for 16 h with tunicamycin) or FcLR5-50 (I, control; K, pre-incubated with tunicamycin) were incubated for 1 h with anti-Fc receptor antibody at 37°C to visualize the transient cell surface appearance (bar, 10 μ m).

cytoplasmic basolateral sorting signals. Serial confocal sections did not show such a widespread tunicamycin-induced redistribution of FcLR5-50 as in the case of the apical chimera (Figure 5C, control; D, tunicamycin). Nevertheless, the immunofluorescence experiments suggested that the intracellular fluorescence became more prominent in tunicamycin-treated cells (compare Figure 5C2 and D2), but this staining was more punctate and did not show significant overlap with the Golgi marker (Figure 5F1, anti-Fc receptor; F2, anti-giantin). Reduced, but still basolateral, cell surface expression after tunicamycin treatment was also observed when the antibody was bound to non-permeabilized cells on ice (not shown).

We incubated subconfluent MDCK cells expressing either FcLR5-22 or FcLR5-50 with the anti-Fc receptor antibody at 37°C, with or without tunicamycin treatment, to test whether the reduction in cell surface expression was due to inhibition of cell surface transport or changed endocytic behavior. In the absence of tunicamycin, similar staining was obtained in cells expressing FcLR5-22 (Figure 5G) or FcLR5-50 (Figure 5I). If tunicamycin-treated cells were analyzed, only a small amount of labeling was observed in cells expressing FcLR5-22 (Figure 5H). In contrast, tunicamycin-treated cells

expressing the chimera containing basolateral sorting signals, FcLR5-50, were still labeled efficiently, indicating that the receptor still reached the cell surface. The intracellular punctate staining was more prominent in tunicamycin-treated cells, suggesting that the intracellular staining observed in the steady state was due to FcLR5-50 chimera that had been internalized from the plasma membrane and that *N*-linked sugars might also affect endocytic trafficking. In the presence of tunicamycin, newly synthesized chimeras were thus only transported to the cell surface if the protein contained the entire cytoplasmic domain of the LDL receptor, indicating that *N*-linked carbohydrates are only important for cell surface transport in the absence of basolateral sorting signals.

We next tested whether *N*-linked carbohydrates would also be important for cell surface transport of Fc receptor chimera in stably transfected CHO fibroblasts. In control CHO cells, staining for FcLR5-22 did not significantly overlap with giantin labeling (Figure 6A1, anti-Fc receptor; 6A2, anti-giantin; the staining in A1 appears hazy since there is only a small amount of intracellular chimera but a strong staining of the plasma membrane). Incubation with tunicamycin, however, resulted in accumulation of FcLR5-22 in a perinuclear structure that co-localized with

the Golgi marker (Figure 6B1, anti-Fc receptor; 6B2, anti-giantin). Additionally, some vesicular structures could also be seen, which were, however, much less pronounced than in cells expressing FcLR5-50 (Figure 6C1, without, and D1, with tunicamycin). Since both chimeras contain the clathrin-coated pit domain of the LDL receptor, these dots are likely to represent endosomes or lysosomes and are therefore less pronounced in cells expressing FcLR5-22 since this chimera is transported only inefficiently to the cell surface. In the case of FcLR5-22, it could also be that a protein that accumulates in the Golgi complex is ultimately delivered to lysosomes due to an autophagocytic process. Importantly, in CHO cells expressing FcLR5-50, no significant accumulation in the area of the Golgi complex was observed in control cells (Figure 6C1, anti-Fc receptor; 6C2, anti-giantin) or in tunicamycin-treated cells (Figure 6D1, anti-Fc receptor; 6D2, anti-giantin). Cell surface transport of chimeric Fc receptors in CHO cells thus requires either the luminal carbohydrates or the entire LDL receptor cytoplasmic domain, which contains two basolateral targeting determinants; hence, the same determinants that control Golgi to cell surface transport in MDCK cells are also active in CHO fibroblasts.

Discussion

The three model membrane proteins that we used to test the effect of *N*-linked carbohydrates on cell surface transport in MDCK cells exhibited the same behavior. Without a cytoplasmic basolateral targeting determinant, the presence of *N*-linked glycans was required for efficient cell surface transport and resulted in apical sorting, indicating that *N*-linked carbohydrates can act as apical targeting signals for membrane proteins. In both cases tested, cytoplasmic basolateral targeting determinants were dominant over the apical targeting capability of the carbohydrates, explaining the surprising apical expression of many mutated basolateral membrane proteins with inactivated basolateral targeting determinants (Rodriguez-Boulant and Powell, 1992; Matter and Mellman, 1994).

Without a cytoplasmic basolateral targeting determinant and apically sorting carbohydrates, our reporter proteins accumulated in the Golgi complex in MDCK and CHO cells, indicating that efficient cell surface transport depends on the same targeting determinants in polarized and non-polarized cells. We made the same observation with three different membrane proteins, one of them a glycoprotein, that have completely different characteristics, function and subcellular localization. Two of the proteins we used, one a cell surface protein and the other an early secretory pathway protein, are normally not glycosylated but are transported from the Golgi complex to the cell surface in a glycosylation-dependent manner; hence, it is difficult to imagine that they were actively retained in the Golgi complex due to a novel quality control mechanism since the ectopic carbohydrates would have had to improve the structure of these normally non-glycosylated proteins. Similarly, non-glycosylated chimeric Fc receptor only accumulated in the Golgi apparatus if it lacked the last 28 amino acids of the cytoplasmic domain of the LDL receptor, which are required for efficient basolateral targeting. It is thus unlikely that glycosylation affected specific

Fig. 6. Differential effect of tunicamycin on the cell surface expression of chimeric Fc receptors in CHO cells. CHO cells stably expressing FcLR5-22 (**A** and **B**) or FcLR5-50 (**C** and **D**) were incubated without (**A** and **C**) or with (**B** and **D**) tunicamycin for 6 h. Cells were then fixed and labeled with anti-Fc receptor (**A1**, **B1**, **C1** and **D1**) and anti-giantin antibodies (**A2**, **B2**, **C2** and **D2**). Shown are confocal sections through the middle of the cells containing the Golgi complex (bar, 5 μ m).

properties of the proteins that are responsible for an active retention in the Golgi complex.

By immunoelectron microscopy, HAoccludinCT3 appeared to be distributed over the entire Golgi complex; hence, it could be that the carbohydrates are also involved in transport through the Golgi complex. Nevertheless, this would have to be a specific requirement for proteins lacking other transport determinants since basolaterally transported occludin and Fc receptor chimera did not accumulate in the Golgi complex in the absence of glycosylation. Alternatively, it could also be that the distribution over the entire Golgi complex is caused by constant recycling of proteins that accumulate in the Golgi complex because they lack a signal for efficient cell surface transport or for transport along another route that departs from the Golgi apparatus. Such a scenario is in agreement with a recently proposed model in which retrograde traffic from the Golgi complex, including the TGN, occurs constantly and can be signal-independent (Cole *et al.*, 1998). Hence, efficient Golgi complex to cell surface transport could only occur in the presence of a cell surface determinant, and the absence of a signal for forward transport would result in a distribution over the entire Golgi complex due to passive retrograde transport.

Accumulation of membrane proteins in the Golgi com-

plex has been observed previously. In fibroblasts, a chimera consisting of human growth hormone and the transmembrane and cytosolic domains of vesicular stomatitis virus (VSV) G protein was shown to accumulate in the Golgi complex if not glycosylated (Guan *et al.*, 1985). Interestingly, this construct accumulated in the Golgi apparatus even though the cytoplasmic domain of VSV G protein is known to contain a basolateral targeting determinant (Thomas *et al.*, 1993). Perhaps the functionality of the cytoplasmic domain determinant requires correct oligomerization, which may explain why it is not active in the chimeric construct.

There are other observations in the literature that support a model in which efficient exit from the Golgi complex is signal-dependent. Mutant asialoglycoprotein receptor H1 subunit that lacks the cytoplasmic domain was shown to accumulate in the Golgi complex (Wahlberg *et al.*, 1995). While this was attributed to steric problems, cell surface transport could be rescued by the cytoplasmic domain of the transferrin receptor, which contains a well-defined basolateral sorting determinant (Odorizzi and Trowbridge, 1997), or by a sequence that contains a leucine-isoleucine motif (Wahlberg *et al.*, 1995). Interestingly, introduction of an arginine residue into the transmembrane domain of influenza virus neuraminidase, which is capable of mediating apical sorting (Kundu *et al.*, 1996), also resulted in accumulation in the Golgi complex, indicating that different types of determinants can mediate efficient Golgi to cell surface transport (Sivasubramanian and Nayak, 1987).

It has been shown previously that a membrane protein that apparently lacks any type of cell surface targeting determinant can be distributed randomly over the entire cell surface (Yeaman *et al.*, 1997). Nevertheless, the efficiency of cell surface transport has not been determined in this case. Therefore, the random cell surface distribution could be due to inefficient cell surface transport to both plasma membrane domains or, alternatively, to secondary interactions with other transported proteins.

N-linked carbohydrates did not mediate cell surface transport with the same efficiency in all three cases that we studied, since a fraction of the glycosylated ERGIC-53 chimera could still be localized to the Golgi complex. Furthermore, accumulation of mutant plasma membrane glycoproteins in the Golgi complex has been observed before (see above). Thus, the efficiency of *N*-linked glycan-mediated cell surface transport varies from glycoprotein to glycoprotein. The parameters that determine the efficiency of cell surface transport mediated by a particular *N*-linked carbohydrate are not known, but may depend on the composition of the oligosaccharide, the environment of the glycosylation site in the peptide (i.e. interactions between the carbohydrates and amino acid residues) or on the distance of the glycan from the membrane.

The mechanism underlying carbohydrate-mediated cell surface transport and apical sorting is not known, but might involve the glycolipid-cholesterol rafts that are thought to sort GPI-anchored proteins and some transmembrane proteins due to special characteristics of their transmembrane domains (Kundu *et al.*, 1996; Simons and Ikonen, 1997). It could be conceivable that *N*-linked glycans interact with rafts by binding to lectin-like molecules similar to ERGIC-53, a mannose-binding protein of

the early secretory pathway (Itin *et al.*, 1996), or VIP-36, an *N*-acetyl galactosamine-binding protein of apical and basolateral transport vesicles (Fiedler and Simons, 1996). It has been shown recently that the *O*-glycosylated stalk domain of neurotrophin receptors is required for apical targeting (Yeaman *et al.*, 1997). While it was not tested whether this requirement is due to the carbohydrates or to a peptide determinant, it could be that proteins can also interact with apically sorting lectins via *O*-linked carbohydrates, which, in contrast to *N*-glycans, contain *N*-acetyl galactosamine and could, therefore, interact with VIP-36. The association of lectins with rafts could then be mediated either by the transmembrane domains of the lectins or by binding to the carbohydrates of the glycolipids, resulting in membrane subdomains in the TGN containing glycolipids, cholesterol, GPI-anchored proteins, and glycosylated membrane and secretory proteins.

The next question then is how such membrane subdomains are incorporated into apical transport vesicles. One possibility is that these membrane subdomains are already sufficient for incorporation into vesicles (Simons and Ikonen, 1997). Alternatively, transmembrane sorting lectins may connect the whole complex (i.e. rafts and transmembrane proteins cross-linked and concentrated by the transmembrane lectins) to a sorting machinery on the cytosolic side of the membrane via their cytoplasmic domains. In such a model, incorporation into apical vesicles would then be driven by a membrane-associated coat complex similar to other sorting steps in the biosynthetic and endocytic transport systems (Mellman, 1996; Schekman and Orci, 1996). Consequently, one would expect there to also be apically sorting cytoplasmic domains. The sensitivity of apical sorting and transport to brefeldin A, a drug that inactivates certain membrane coats involved in protein sorting, would indeed suggest the involvement of such a cytosolic sorting machinery (Low *et al.*, 1992; Matter *et al.*, 1993). A model in which transmembrane lectins and not glycolipid-cholesterol rafts are responsible for incorporation into apical transport vesicles would also explain how apical transmembrane proteins can be sorted normally while GPI-anchored proteins are transported preferentially to the basolateral membrane in FRT cells (Zurzolo *et al.*, 1994; Weimbs *et al.*, 1997).

One could even imagine that glycolipid-cholesterol rafts and *N*-linked glycans mediate apical sorting independently of each other. In the intestinal epithelial cell line Caco-2, apical transport can occur by a direct pathway from the Golgi complex to the apical cell surface and by an indirect pathway via the basolateral plasma membrane domain (Le Bivic *et al.*, 1990; Matter *et al.*, 1990). The extent of direct apical sorting differs from one protein to another. For example, sucrase-isomaltase is preferentially sorted into the direct pathway, while other proteins like dipeptidylpeptidase IV and aminopeptidase N are transported along both pathways. Sucrase-isomaltase, but none of the peptidases, becomes detergent-insoluble during transit through the Golgi, an assay often used as an indication of association with glycolipid-cholesterol rafts (Garcia *et al.*, 1993; Mirre *et al.*, 1996). Thus, it could be that targeting to the apical cell surface domain is mediated by two independent mechanisms, one involving association with glycolipid-cholesterol rafts and the other with *N*-

linked glycans, and that the capacities and/or the directionality of these pathways vary from one epithelial tissue to another, leading to the plasticity of apical membrane protein sorting in different epithelial cell types.

Generally, basolateral targeting appears to be dominant over apical sorting. Many basolateral membrane proteins are targeted efficiently to the apical membrane when their basolateral targeting determinant is inactivated (Matter and Mellman, 1994). Even influenza virus HA, an apically sorted *N*-glycosylated protein that possesses a transmembrane domain able to associate with glycolipid-cholesterol rafts (Scheiffele *et al.*, 1997), is sorted to the basolateral membrane when a tyrosine-dependent basolateral targeting determinant is introduced into its cytoplasmic domain (Brewer and Roth, 1991). One possibility to explain this dominance would be to assume that the basolateral determinants have a higher affinity than the apical targeting signals for their respective sorting machineries. A simpler mechanism would be that the sites for apical and basolateral sorting are spatially separated so that proteins encounter the site of basolateral sorting before that of apical sorting.

To summarize, membrane proteins lacking a signal for forward transport accumulate in the Golgi apparatus and are delivered only inefficiently to the plasma membrane, indicating that efficient exit from the Golgi complex is signal-mediated. Since efficient exit from the endoplasmic reticulum also appears to be signal-dependent (Kappeler *et al.*, 1997; Nishimura and Balch, 1997), this indicates that membrane proteins are not transported efficiently along the secretory pathway to the cell surface by default. Moreover, a protein possessing neither a signal for leaving the Golgi apparatus nor a determinant for a specific subcompartment of the Golgi complex may distribute over the entire Golgi complex due to passive retrograde transport (Cole *et al.*, 1998). Exit from the Golgi complex appears to be mediated by different types of signals ranging from cytoplasmic targeting determinants, particular transmembrane domains and GPI anchors, to glycans in the luminal domains of membrane proteins. Because of the sorting patterns in different tissues, these determinants may represent independent sorting pathways that exist in polarized and non-polarized cells and that are directed to particular cell surface domains in a manner that depends on the particular needs of a given cell type.

Materials and methods

Cell lines, cDNAs and transfections

MDCK and CHO cells were cultured and transfected as described (Matter *et al.*, 1992; Miettinen *et al.*, 1992). Tunicamycin was prepared as a 10 mg/ml stock in 25 mM NaOH and then diluted into culture medium to a final concentration of 12 µg/ml. Cell lines expressing HAoccludin, HAoccludinGL1+2, HAoccludinCT3, FcLR5-22 and FcLR5-50 were described previously (Matter *et al.*, 1992; Balda *et al.*, 1996a). Glycosylation sites were introduced into the cDNA of HAoccludinCT3 at the same sites as in HAoccludin (Matter and Balda, 1998a). The ERGIC-53 mutants MECA and MECAG were constructed as described (Itin *et al.*, 1995). For both chimeric constructs as well as for ERGIC-53, a myc tag was introduced after the signal sequence. Using PCR-based splicing and mutagenesis, the transmembrane domain of ERGIC-53 was replaced with that of CD4 and the cytosolic domain was substituted by a sequence coding for RRAAAAAAAAAA. To generate MECAG, two artificial *N*-glycosylation sites were introduced in the luminal domain near the N-terminus: the aspartic acid at position 61 was changed to an asparagine (Itin *et al.*, 1995), and an *N*-glycosylation

tag, VNATASA, was introduced at the unique *Sac*II site immediately after the myc tag. All constructs were cloned into pCB6 for transfection.

Immunofluorescence, confocal microscopy and electron microscopy

Cells grown on coverslips for 3 days or on Transwell filters for at least 5 days were fixed for 20 min in 3% paraformaldehyde and then permeabilized with 0.1% saponin in phosphate-buffered saline (PBS) in the presence of 10 mM glycine and 0.5% bovine serum albumin (BSA). After 15 min, the cells were incubated with primary antibodies and processed further for confocal microscopy (Balda *et al.*, 1996a). For cell surface labeling, antibody diluted in cold PBS⁺-BSA (PBS containing 0.5% BSA and 0.5 mM CaCl₂) was added to filter-grown MDCK cells on ice (Matter and Balda, 1998a). After 2 h, cells were washed four times with cold PBS⁺-BSA and then fixed and permeabilized with paraformaldehyde and saponin as above. Occludin constructs were visualized with either the monoclonal or the polyclonal anti-HA antibody (Daro *et al.*, 1996), ERGIC-53 chimera with monoclonal antibody G1/93 (Schweizer *et al.*, 1988), giantin with monoclonal antibody G1/133 (Linstedt and Hauri, 1993), and Fc receptor chimera with a polyclonal antibody (Green *et al.*, 1985). The samples were analyzed with a Zeiss confocal laser scanning microscope (LSM 410 invert) equipped with an argon and a helium-neon laser for double fluorescence at 488 and 543 nm (emission filters: BP510-525 and LP590) using a 63× Apochromat lens. For electron microscopy, the pre-embedding labeling of HAoccludinCT3-expressing cells was done exactly as described using the monoclonal anti-HA antibody (Balda *et al.*, 1996a).

Protein analysis

For immunoblots of total cell extracts, cells from 12-well dishes were lysed directly in 500 µl of extraction buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.2% Na-dodecylsulfate, 1% Empigen BB and 40 µg/ml phenylmethylsulfonyl fluoride). After a 10 min spin in a microfuge, proteins were extracted from the supernatant and precipitated with methanol and chloroform (Wessel and Flügge, 1984). The samples were transferred to nitrocellulose after the electrophoretic separation, and the membranes were probed with the monoclonal anti-HA antibody for occludin mutants (Daro *et al.*, 1996), a monoclonal anti-myc antibody for ERGIC-53 chimera (Evan *et al.*, 1985) or the polyclonal anti-Fc receptor antibody for FcLR chimera (Green *et al.*, 1985). Incubations with primary and secondary antibodies as well as the detection with enhanced chemiluminescence (ECL) were as described previously (Balda *et al.*, 1996a).

To monitor the cell surface appearance of newly synthesized protein, filter-grown MDCK cells were metabolically labeled with [³⁵S]methionine/cysteine (Amersham) (Matter *et al.*, 1992). After completion of the pulse-chase protocol, the cells were cooled on ice and either the apical or basolateral cell surface was biotinylated with biotin-hydrazide (Pierce) (Braendli *et al.*, 1990). Cells were then solubilized with extraction buffer, and Fc receptor chimera or occludin mutants were immunoprecipitated with anti-Fc receptor antibody or anti-HA antibody, respectively (Matter *et al.*, 1993). Immunocomplexes were dissociated and biotinylated proteins were precipitated with streptavidin-agarose. Precipitates were then analyzed by SDS-PAGE (6–18% gradient gels) and fluorography (Matter *et al.*, 1992).

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