N-glycans are not a universal signal for apical sorting of secretory proteins

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Abstract In MDCK cells, *N*-glycans have been shown to determine the sorting of secretory proteins and membrane proteins to the apical domain in the absence of a dominant basolateral targeting signal. We have examined the sorting of endogenous proteins in ECV304 cells in the presence and absence of tunicamycin, an inhibitor of *N*-linked glycosylation. A prominent apically secreted protein of 71 kDa was not *N*-glycosylated and continued to be secreted apically in the presence of tunicamycin. In contrast, other endogenous proteins that were *N*-glycosylated were secreted preferentially into the basolateral medium or without polarity. When rat growth hormone was expressed in MDCK and ECV304 cells, we observed 65 and 94% of the secretion to the basolateral medium, respectively. Introduction of a single *N*-glycan caused 83% of the growth hormone to be secreted at the apical surface in MDCK cells but had no significant effect on the polarity of secretion of growth hormone in ECV304 cells. These results indicate that not all cell lines recognise *N*-glycans as a signal for apical sorting and raises the possibility of using ECV304 cells as a model system for analysis of apical sorting molecules.

Key words: Apical targeting; *N*-glycan; Growth hormone; Secretion; Sorting

1. Introduction

Basolateral sorting of membrane proteins in epithelial cells has been found to be encoded by sequences on their cytoplasmic domains that frequently, but not always, involve tyrosine motifs [1–4]. Sorting is presumed to occur by interaction of these motifs with cytosolic coat proteins involved in the membrane traffic. The different lipid composition of the apical plasma membrane of epithelial cells compared to the basolateral plasma membrane initially suggested that targeting of proteins to the apical surface might be the result of an affinity of apical proteins for glycosphingolipid rafts formed in the Golgi and transported to the apical plasma membrane [5,6]. This hypothesis explained the sorting of some glycosyl-phosphatidylinositol (gpi)-anchored proteins to the apical surface [7,8] and concentrating of some glycolipids on the apical domain [9]. Since no part of a gpi-linked protein extends into the cytosol, an interaction with cytosolic coat proteins is not possible. Their attachment to membranes via the saturated fatty acyl chains of an inositol lipid, however, could account for their affinity for glycosphingolipid rafts [6,7]. A second signal for apical targeting has been implicated by the observation that deletion or mutation of the basolateral targeting motif of several receptors causes these proteins to be sorted to the apical surface of epithelial cells suggesting that a cryptic apical targeting signal is present in the extracellular domain of these proteins [10,11]. It has recently been suggested that this apical targeting is due to the presence of *N*-glycans on the extracytoplasmic domain that interact with lectins found in the apically sorted lipid rafts [12]. This hypothesis was derived from examination of the polarity of secreted endogenous proteins in epithelial cells. Proteins that contained *N*-glycans were sorted more efficiently to the apical surface than those which were not glycosylated. Proof came from the introduction of consensus sites for *N*-glycosylation into rat growth hormone (GH), a protein that is normally not glycosylated. As increasing numbers of ^{N}-glycosylation sites were introduced into the molecule, the secretion became increasingly polarised to the apical surface [13]. Interestingly, an animal lectin, VIP36, with sequences homologous to leguminous lectins was identified in apically directed secretory vesicles [14]. However, there is no direct evidence yet for the functioning of the VIP36 protein as an animal sorting lectin in vivo.

We have studied the sorting of secretory proteins in ECV304 cells. This cell line was described as a spontaneously transformed human umbilical vein endothelial cell line, however, it has recently been shown that this cell line is in fact a human bladder cancer epithelial cell line closely related to T-24. Previous experiments have shown that ECV304 cells form monolayers when cultured on Transwell inserts and normally recognise the basolateral targeting signal of E-cadherin and apical polarity signal of haemagglutinin from influenza virus [15], but do not efficiently recognise the basolateral targeting signal of the polymeric immunoglobulin receptor [16]. In this paper, we have used this cell line to determine the universality of *N*-glycans as a sorting determinant of secretory proteins.

2. Materials and methods

2.1. Cell culture

ECV304 cells and type II MDCK cells were cultured in M199 and DMEM supplemented with 10% FCS. The cells were seeded at a density of 1×10^6 cells per 4.5 cm^2 of Transwell filter (Corning Costar, Cambridge, MA, USA) for ECV304 cells and 1.8×10^6 cells per filter for MDCK cells. The cells were then grown for 3 days with fresh medium each day. For metabolic labelling, the cells were first depleted of endogenous pools of methionine and cysteine by incubation for 30 min in serum-free DMEM lacking these two amino acids. The culture medium was then supplemented with an EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Germany) and 0.3 mCi/ml of L-[^{35}S]Pro-mix (Amersham Life Science, UK). After 2 h incubation at 37°C, the apical and basolateral media were collected, centrifuged

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to remove any cell debris and secreted proteins concentrated onto 15 μl of StrataClean resin (Stratagene).

2.2. Protein N-glycanase F (PNGase F) digestions

Apical or basolateral media of ECV304 cells were prepared and boiled in 0.1% SDS and 50 mM DTT for 5 min to denature the proteins. NP40 was then added to a final concentration of 0.75% and the proteins were digested with 1 U/ml of PNGase F for 16 h at 37°C as indicated. The secretory proteins were then concentrated onto StrataClean resin and separated by 10% SDS-PAGE. To digest individual secretory proteins, 2 mm wide slices of the SDS-PAGE gel were incubated in 0.1% SDS, overnight, before treatment with PNGase F as above.

2.3. Transfection and expression of GH constructs

ECV304 cells were transfected with GH cDNA constructs under the control of the CMV promoter using lipofectamine reagent and stable colonies were selected with 0.6 mg/ml G418. 1.0×10^6 cells were plated per filter and grown for 3 days in M199 medium containing 10% FCS. Cells were then induced with 2 mM sodium butyrate for 16 h, washed three times with PBS and incubated in DMEM at 37°C, 5% CO_2, for 3 h. The medium from the apical and basolateral chambers was collected and the secreted protein concentrated on StrataClean resin. All of the protein sample was loaded onto a 12.5% SDS-PAGE gel and transferred to nitrocellulose. Proteins were detected by Western blotting with rat GH rabbit polyclonal antiserum (Chemicon), followed by anti-rabbit horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence (ECL) (Amersham).

3. Results

Polarised secretion of proteins in ECV304 cells was studied using monolayers of cells grown on Transwell filters for 3 days and metabolically labelled for 2 h with 300 μCi/ml L-[^{35}S]methionine and cysteine. The secreted proteins were then concentrated from the apical and basolateral media and separated by SDS-PAGE. Several proteins are secreted with a distinct polarity under these conditions. In Fig. 1A, it can be seen that protein 1 (71 kDa) is secreted with a pronounced apical polarity, whereas protein 2 (57 kDa) is predominantly basolateral. We have also identified, in Fig. 1A, a prominent non-polarised secretory protein, labelled protein 3

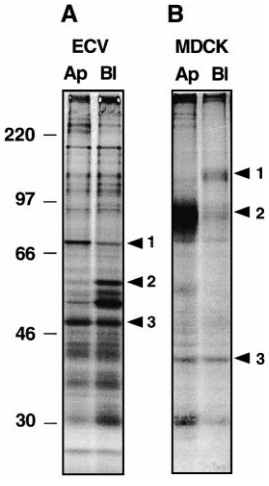

Fig. 1. Polarity of secretory proteins in ECV304 and MDCK cells. (A) and (B) show a phosphorimage of the labelled proteins from ECV304 and MDCK cells, respectively, after separation on 10% SDS-PAGE. Ap = apical, Bl = basolateral. The MDCK secretory proteins were run on a non-reducing gel to preserve the integrity of gp80 (protein 2). (A) was obtained under reducing conditions. For comparison, a non-reducing gel of ECV304 secretory proteins is shown in Fig. 3B.

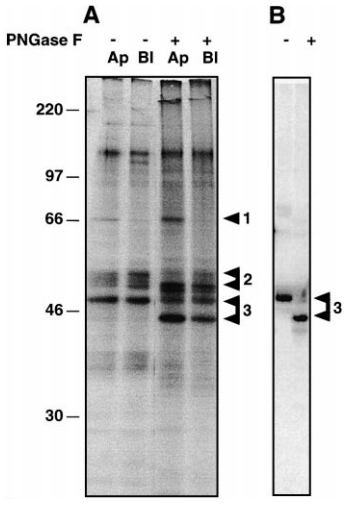

Fig. 2. Identification of *N*-glycosylated secretory proteins. (A) Metabolically labelled secretory proteins from apical or basolateral media of ECV304 cells before and after treatment with PNGase F, (B) protein 3 before and after treatment with PNGase F.

(48 kDa). The degree of apical secretion of these proteins was previously measured as 82 ± 5%, 14 ± 3% and 50 ± 1%, respectively [16]. For comparison, it can be seen that MDCK cells, which have been widely used as a model for epithelial cell sorting, also secrete proteins with varying degrees of polarity (Fig. 1B) including a basolaterally secreted protein of 114 kDa (protein 1), a major apically secreted protein of 80 kDa (protein 2) on non-reducing gels which has been described as clusterin or apo J and some proteins which are non-polarised (e.g. protein 3). The 80 kDa protein has shown to be secreted randomly in tunicamycin-treated cells consistent with the hypothesis that *N*-glycans are necessary for apical secretion in epithelial cells [17].

The presence of *N*-linked glycans on the proteins secreted by ECV304 cells was determined by treatment of the media with PNGase F prior to running the SDS-PAGE (Fig. 2A). PNGase F cleaves the bond between the asparagine residue on the proteins and the first *N*-acetyl glucosamine of the glycan, releasing the complete *N*-glycan from the protein. In Fig. 2A, it can be seen that protein 1 migrated with an identical mobility before and after treatment with PNGase F. However, both proteins 2 and 3 were substantially shifted in mobility by the PNGase F treatment showing that these proteins are *N*-glycosylated. The assignment of bands after PNGase F treatment was determined by cutting out strips from one SDS gel, eluting the proteins from the strips and treating this eluate with PNGase F. In Fig. 2B, it can be seen that the 48 kDa protein 3 shifts to an apparent mobility of 40 kDa after PNGase F treatment verifying the identification of the bands corresponding to protein 3 in Fig. 2A.

Having established which secreted proteins were post-translationally modified with *N*-glycans, we then examined the secretion of these proteins in ECV304 cells treated with tunicamycin which inhibits the attachment of *N*-glycans during the synthesis of the proteins in the endoplasmic reticulum. In Fig. 3A, it can be seen that protein 1, which does not contain any *N*-glycans, is similarly sorted to the apical medium in the presence or absence of tunicamycin. On a non-reduced SDS polyacrylamide gel, protein 1 runs slightly faster than on a reduced gel indicating the presence of one or more disulfide

bonds, but ruling out the possibility that this protein is part of a disulfide-linked multiprotein complex. Thus, apical sorting is unlikely to be due to an associated *N*-glycosylated protein. Protein 2 which is secreted predominantly to the basolateral medium contains an *N*-linked glycan and is similarly sorted in either the presence or absence of tunicamycin. However, protein 2 is not present on non-reducing gels (Fig. 3B), showing that it must be part of a disulfide-linked multi subunit protein complex. Protein 3 was not detectably polarised despite the presence of *N*-glycans. In tunicamycin-treated cells, it migrates with the same apparent mobility (Mr = 40 kDa) as PNGase F-treated protein 3, showing that it has not been glycosylated as it passed through the secretory system. Despite this, the unglycosylated form of protein 3 is similarly sorted as glycosylated protein 3 with equal amounts secreted to both apical and basolateral domains (Fig. 3A). On the non-reducing SDS-PAGE (Fig. 3B), protein 3 migrated with exactly the same mobility as on reduced gels indicating that this protein is not disulfide-linked to other proteins in the cell and contains no disulfide bonds. Thus, for protein 3, the presence of *N*-glycans did not increase the apical sorting and for protein 1, apical sorting was achieved without *N*-glycans.

In order to verify this conclusion, we transfected ECV304 cells with the same constructs encoding human GH [18] as were previously used by Schieffele et al., except that the expression plasmid used was pBKCMV (Clontech). Wild-type GH has no sites for *N*-linked glycosylation (GH0), whereas the recombinant GH constructs have a site for *N*-linked glycosylation engineered into the sequence at either position 14 (GH1) or 95 (GH2) or at both positions 14 and 95 (GH1,2). Sufficient GH was secreted by the cell lines in the presence of 2 mM butyrate to be detected by a Western blot with ECL detection (Fig. 4). As previously reported [13], we observed two bands from the GH1 and GH2 constructs corresponding to the *N*-glycosylated form of GH and residual unglycosylated GH (Fig. 4A). In GH1 and GH2, the glycosylated proportions were between 50 and 60%, reflecting a similar access to glycosyl transferases by both proteins. The GH construct with both sites modified, GH1,2, gives rise to a diffuse band corresponding to approximately 25% of the expressed GH. Quantitation of the bands corresponding to GH secreted apically and basolaterally from three different Transwell filters in different experiments showed that the basolateral sorting of GH from GH0, GH1 and GH2 expressing cells was 94.8 ± 2.3%, 93.9 ± 1% and 83.6 ± 2% ($n = 3$) of the total, respectively. Thus, addition of the *N*-glycan in position 14 (GH1) had no effect on the polarity of secretion while that at position 95 only increased apical secretion by 11%. For comparison, we also transfected MDCK cells with the identical constructs. The unglycosylated GH0 construct showed 65 ± 2% basolateral secretion. However, addition of a single glycan caused 83 ± 6% ($n = 3$) of the GH to be secreted from the apical surface leaving only 17% basolateral (Fig. 4B). These figures are close to those observed by Schiefele et al. [13]. Thus, addition of a single glycan is a powerful apical targeting determinant in MDCK cells but has little effect in ECV304 cells. It can also be seen in Fig. 4 that GH glycosylation mutants that escaped glycosylation (i.e. the 26 kDa band in GH1, GH2 and GH1,2) were secreted with a similar polarity to the glycosylated protein, indicating the formation of dimers or multimers between glycosylated and unglycosylated GH as has been described previously [13].

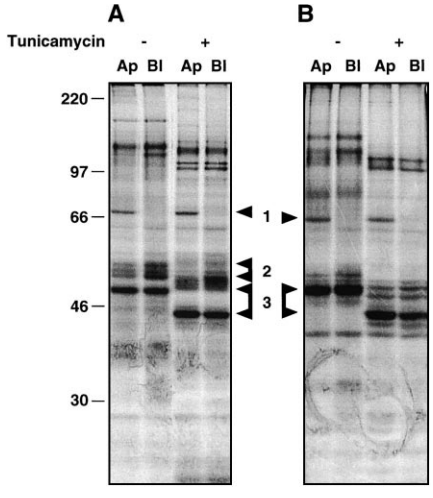

Fig. 3. Sorting of unglycosylated secretory proteins in ECV304 cells. Metabolically labelled secretory proteins were isolated from control cells or cells pre-treated with 10 μg/ml of tunicamycin for 2 h prior to the metabolic labelling. The proteins were concentrated on StrataClean resin and separated by (A) reducing or (B) non-reducing SDS-PAGE.

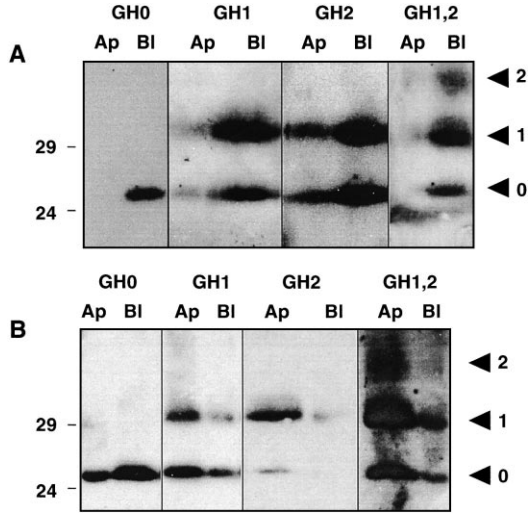

Fig. 4. Sorting of GH in transfected ECV304 cell lines. (A) shows a Western blot of the GH secreted by 1.0×10^6 ECV304 cells transfected with unglycosylated GH0, mono-glycosylated GH1 and GH2 and di-glycosylated GH1,2. (B) shows a Western blot of MDCK cells transfected with GH0, GH1, GH2 and GH1,2 under the same conditions as (A). The arrowheads marked 0, 1 and 2 mark the mobilities of GH with zero, one and two glycans. Similar results were obtained with more than one clone from two separate transfections.

4. Discussion

MDCK cells have been extensively used as a model of sorting, largely due to their ability to form highly impermeable monolayers on Transwell filters. When studies have been performed on other polarised cells, different results have sometimes been described suggesting that sorting is modulated according to the physiology of the cell. For example, in MDCK cells, all sorting of membrane proteins for apical and basolateral domains occurs as the proteins leave the Golgi [19]. However, in hepatocytes, both apical and basolateral proteins are delivered to the basolateral surface, apical proteins are

then sorted away from basolateral proteins in endosomes and transcytosed to the apical surface [20].

We initially chose to study ECV304 cells because they were described as endothelial cells, which might be expected to have quite different apical sorting processes from epithelial cells as their apical surface faces the blood supply. However, it has recently been revealed that the microsatellite fingerprint of ECV304 cells is consistent with them being a variant of T-24 human bladder cancer cells. Nevertheless, several laboratories have described the ability of these cells to form monolayers [21,22] and sort apical and basolateral membrane proteins [15]. In this paper, we have shown that ECV304 cells are active secretory cells that can secrete proteins with a marked polarity. Surprisingly, the most pronounced apically secreted protein was not *N*-glycosylated, while other proteins which were *N*-glycosylated were not secreted apically, indicating that mechanisms other than *N*-glycosylation must exist for the apical sorting of secretory proteins in these cells. When both ECV304 cells and MDCK cells were transfected with mutants of GH having none, one or two sites for *N*-glycosylation, it was found that *N*-glycosylation promoted apical sorting in MDCK cells as previously described. In ECV304 cells, however, GH was secreted predominantly to the basolateral medium, independent of the number of *N*-glycans. We cannot conclude, however, that the ECV304 cells grow in an inverted manner on Transwell filters since it has previously been shown that haemagglutinin and E-cadherin are normally sorted to the apical membrane (facing the medium) and basolateral membrane (facing the filter), respectively [15]. Thus, it appears that ECV304 cells have lost the ability to distinguish *N*-glycans as a sorting signal. When we performed RT-PCR assays for VIP36, we found a lectin in the TGN of MDCK cells that was postulated as a possible sorting molecule. We found that it was absent from the ECV304 cells even though the primers used would have hybridised equally with dog and human VIP36 sequences (data not shown). Thus, it could be that transformation of the ECV304 cells resulted in loss of the sorting mechanism. ECV304 cells therefore provide a model in which the hypothesis that VIP36 has a role in apical sorting can be tested.

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References

[1] Hunziger, W., Harter, C., Matter, K. and Mellman, I. (1991) Cell 66, 1–20.
[2] Matter, K. and Mellman, I. (1994) Curr. Opin. Cell. Biol. 6, 545–554.
[3] Keller, P. and Simons, K. (1997) J. Cell Sci. 110, 3001–3009.
[4] Aroeti, B. and Mostov, K.E. (1994) EMBO J. 13, 2297–2304.
[5] Rodriguez-Boulan, E. and Powell, S.K. (1992) Annu. Rev. Cell Biol. 8, 395–427.
[6] Simons, K. and Ikonen, E. (1997) Nature 387, 569–572.
[7] Brown, D.A., Crise, B. and Rose, J.K. (1989) Science 245, 1499–1501.
[8] Lisanti, M.P., Caras, I.W., Davitz, M.A. and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2145–2156.
[9] Hansson, G.C., Simons, K. and van Meer, G. (1986) EMBO J. 5, 483–489.
[10] Corbeil, D., Boileau, G., Lemay, G. and Cine, P. (1992) J. Biol. Chem. 267, 2798–2801.
[11] Vogel, L.K., Spiess, M., Sjöström, H. and Norén, O. (1992) J. Biol. Chem. 267, 2794–2797.
[12] Fiedler, K. and Simons, K. (1995) Cell 81, 309–312.
[13] Scheiffele, P., Peränen, J. and Simons, K. (1995) Nature 378, 96–98.
[14] Fielder, K. and Simons, K. (1995) J. Cell Sci. 109, 271–276.
[15] Haller, C., Kiessling, F. and Kubler, W. (1998) Eur. J. Cell Biol. 75, 353–361.
[16] Su, T. and Stanley, K.K. (1998) J. Cell Sci. 111, 1197–1206.
[17] Urban, J., Parcyzk, K., Leutz, A., Kayne, M. and Kondor-Koch, C. (1987) J. Cell Biol. 105, 2735–2743.
[18] Guan, J.-L., Machamer, C.E. and Rose, J.K. (1985) Cell 42, 489–496.
[19] Matlin, K.S. (1986) J. Cell Biol. 103, 2565–2568.
[20] Bartles, J.R., Feracci, H.M., Stieger, B. and Hubbard, A. (1987) J. Cell. Biol. 105, 1241–1251.
[21] Takahashi, K., Sawasaki, Y., Hata, J.I., Mukai, K. and Goto, T. (1990) In Vitro Cell Dev. 25, 265–274.
[22] Jakob, R. (1993) J. Med. Microbiol. 39, 26–32.