N-glycans are not the signal for apical sorting of corticosteroid binding globulin in MDCK cells

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Abstract It has been suggested that *N*-glycans act as a general sorting signal for secretory proteins in MDCK cells [Scheiffele et al. (1995) Nature 378, 96–98]. Human corticosteroid binding globulin contains six consensus sites for *N*-glycosylation and is known to be secreted to the apical side of MDCK cells. Our results show that wild-type corticosteroid binding globulin is *N*-glycosylated when it is recombinantly expressed in MDCK cells. Six mutants, each lacking one of the *N*-glycosylation sites, and a mutant lacking all six *N*-glycosylation sites were also secreted to the apical side of MDCK cells in a polarized manner. Thus, the *N*-glycans on corticosteroid binding globulin do not act as an apical sorting signal in MDCK cells.

Key words: MDCK cell; *N*-glycosylation; Apical secretion; Corticosteroid binding globulin

1. Introduction

Polarized epithelial cells are able to secrete proteins specifically at the apical or basolateral plasma membrane. The polarized cell line MDCK, for example, secretes clusterin (gp80) [1], erythropoietin [2] and corticosteroid binding globulin (CBG) [3] mainly at the apical side whereas other secretory proteins are secreted approximately equally at both plasma membrane domains [4–7]. It was recently suggested that *N*-linked glycans act as an apical targeting signal for secretory proteins, based on the observation that growth hormone, normally secreted randomly from MDCK cells, is secreted mainly from the apical side after insertion of one or two *N*-linked glycosylation consensus sites [8]. In addition, erythropoietin is normally secreted to the apical side of MDCK cells, but it is secreted in a non-polarized manner from MDCK cells after mutagenesis of two distinct *N*-linked glycosylation consensus sequences [2]. Similarly, gp80, CBG and *N*-glycosylated growth hormone are all secreted mainly from the apical side of polarized MDCK cells, but after tunicamycin treatment, are secreted in a non-polarized way [1,3,8]. It has also been suggested that *N*-glycans might act as determinants for transport of transmembrane proteins from the trans Golgi network to the apical plasma membrane [9].

The present study aims at investigating the role of *N*-glycosylation in the apical secretion of CBG from MDCK cells. Surprisingly, we found that elimination of the *N*-glycosylation sites in CBG either individually or collectively does not affect its apical secretion.

2. Materials and methods

2.1. Cell culture

MDCK cells (strain II), a kind gift from K. Mostov (University of California), were maintained and transfected as previously described [5]. For sorting experiments, 10^6 cells per well were seeded onto Transwell filters (Costar Europe, Badhoevedorp, The Netherlands: pore size 0.4 μm, diameter 24.5 mm) allowing separate access to the apical and basolateral membranes. Filters were used for experiments 1–2 days after confluence as indicated by assessing the tightness of the monolayer [10]. Under these conditions, monolayers have a transepithelial resistance of ~450 Ω/cm^2, measured by a Millicell-ERS volt-ohmmeter (Millipore Continental Water Systems, Bedford, MA, USA).

2.2. Antibodies and DNA constructs

A rabbit anti-human CBG antiserum [11] was used for immunoprecipitation of wild-type and mutant CBG. The pRc/CMV vector containing cDNA encoding either wild-type human CBG or different CBG *N*-glycosylation mutants [12] was used to generate stable MDCK clones expressing CBG.

2.3. Metabolic labelling of cells and immunoprecipitation

Confluent MDCK cells seeded on filters were pre-incubated for 30 min in methionine-free media and thereafter pulse-labelled with 1 ml labelling medium (MEM without methionine) containing 660 μCi of [^{35}S]methionine applied to the basolateral chamber. After a 20 min pulse, the medium was removed and 1 ml of chase medium (MEM with 10× methionine concentration) was applied to both the apical and basolateral chambers. After 4 h chase, the apical and basolateral media were collected and used for immunoprecipitation. Immunoprecipitation was performed as described [5] and the immunoprecipitated CBG was analyzed using NuPAGE gels (Novex, San Diego, CA, USA).

2.4. Deglycosylation assays

A *N*-glycosidase F deglycosylation kit (Boehringer Mannheim, Mannheim, Germany) was used to study *N*-glycosylation of CBG. Tunicamycin was dissolved in DMSO and stored at −20°C and added to the labelling and chase medium to a final concentration of 15 μg/ml. Control cells were treated with similar concentrations of DMSO.

3. Results and discussion

3.1. CBG expressed in MDCK cells is N-glycosylated

We have used site-directed mutagenesis and recombinant expression to investigate the role of *N*-glycans in the apical secretion of CBG, expressed in MDCK cells. A series of six mutants each lacking one of the six *N*-glycosylation consensus sites and a mutant lacking all six *N*-glycosylation consensus sites were investigated (Fig. 1). All of these mutants have previously been expressed in CHO cells in order to determine the usage of individual *N*-glycosylation sites [12–14]. Clones of

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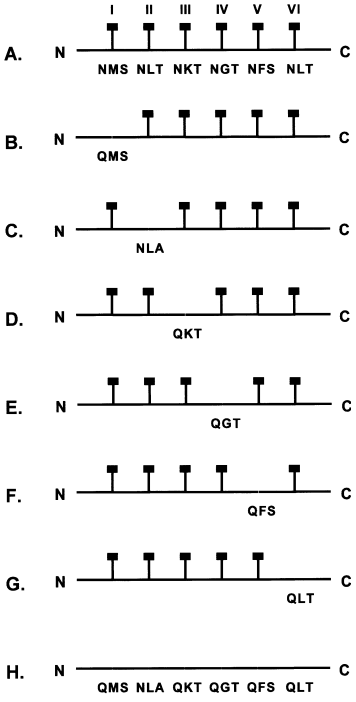

Fig. 1. *N*-glycosylation sites in CBG mutants. The location of consensus sites for *N*-glycosylation in wild-type (A) and mutant proteins (B–H).

cells that showed expression of the desired proteins were identified after transfection of MDCK cells with a mammalian expression vector containing a cDNA encoding each of the seven mutant or wild-type CBG. For each construct, a high- and a low-expressing clone were chosen for further investigation.

To determine whether CBG expressed in MDCK cells is *N*-glycosylated, we used an immunoprecipitation assay. Transfected cells were pulsed for 20 min with [^{35}S]methionine and chased for 4 h. Media were collected, immunoprecipitated, incubated with *N*-glycosidase F and analyzed on NuPAGE

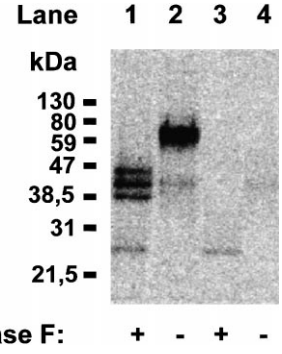

Fig. 2. CBG expressed in MDCK cells is *N*-glycosylated. MDCK cells were labelled with [^{35}S]methionine, chased for 4 h and the media were immunoprecipitated. The immunoprecipitates were treated or not treated with *N*-glycosidase F (NGase F) and analyzed on NuPAGE gels under reducing conditions. *N*-glycosidase F-treated immunopreciptate from cells expressing wild-type CBG (lane 1) shows multiple bands (probably due to residual *N*-glycans) with a clearly higher mobility than untreated immunoprecipitate from the same cells (lane 2). This shows that wild-type CBG is *N*-glycosylated. *N*-glycosidase F-treated (lane 3) and untreated (lane 4) immunoprecipitate from untransfected MDCK cells are shown as a control. The positions of molecular weight standards are indicated.

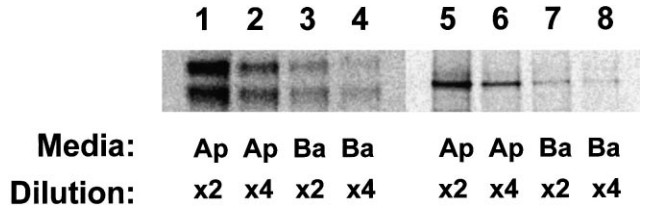

Fig. 3. Immunoprecipitation of CBG is quantitative. MDCK cells expressing wild-type CBG (lane 1–4) or CBG mutated in all six *N*-glycosylation consensus sites (lane 5–8) were grown to confluence on filters. The cells were then labelled for 20 min with [^{35}S]methionine and chased for 4 h and apical (Ap) or basolateral (Ba) media were collected and individually immunoprecipitated in various dilutions (in this case $\times 2$ and $\times 4$) with a fixed amount of antibodies. The immunoprecipitates were analyzed on NuPAGE gels with our prior reduction, since this gave a more condensed appearance of the heterogenously glycosylated CBG. Only when the dilution resulted in a corresponding reduction in the signal (as in the experiment shown), it was assumed that the immunoprecipitation was quantitative and the result was quantitated using a phosphoimager.

gels under reducing conditions. The mobility of wild-type CBG not treated with *N*-glycosidase F (Fig. 2,lane 2) suggests an apparent molecular weight between 50 and 70 kDa. The heterogenous mobility suggests that some of the consensus sites are only partially utilized resulting in populations of molecules with slightly different molecular weights as has also been observed in CHO cells [13]. Wild-type CBG treated with *N*-glycosidase F (Fig. 2, lane 1) also had a heterogenous mobility probably because occasionally, one or two *N*-glycans were not removed by the *N*-glycosidase F treatment. However, the *N*-glycosidase F-treated wild-type CBG has a clearly higher mobility than a similar untreated sample (compare Fig. 2, lane 1 and 2) showing that CBG expressed in MDCK cells is *N*-glycosylated. Immunoreactive material isolated from media from untransfected cells treated with *N*-glycosidase F (lane 3) or non-treated (lane 4) is shown for comparison.

3.2. N-Glycosylation of CBG does not determine its apical secretion from MDCK cells

MDCK clones expressing the different CBG mutants or wild-type CBG were grown to confluence on Transwell filters. The cells were pulsed for 20 min with [^{35}S]methionine and chased for 4 h. The apical and basolateral media were collected, immunoprecipitated and analyzed on NuPAGE gels. Each sample was diluted by a series of 2-fold dilutions and each dilution was immunoprecipitated seperately and analyzed on NuPAGE gels. It was assumed that the immunoprecipitation was quantitative and the result was quantitated using a phosphoimager, only if the 2-fold dilution also resulted in a 2-fold reduction of the signal (Fig. 3).

As shown in Fig. 4A, wild-type CBG is secreted in a polarized way from MDCK cells at an apical/basolateral ratio of 82/18 (S.D. $\pm$ 5: $n = 6$), as previously shown [3]. The deletion of any one of the six *N*-glycosylation sites did not change this apical distribution (Fig. 4B–G) showing that any single *N*-glycan is not essential for the apical delivery of CBG. The CBG mutant lacking all six *N*-glycosylation sites was also targeted to the apical side in a manner indistinguishable from wild-type CBG. This clearly demonstrates that *N*-glycosylation does not play a role as an apical targeting signal for CBG in MDCK cells. Each construct was investigated at least four times, independently of each other and similar results

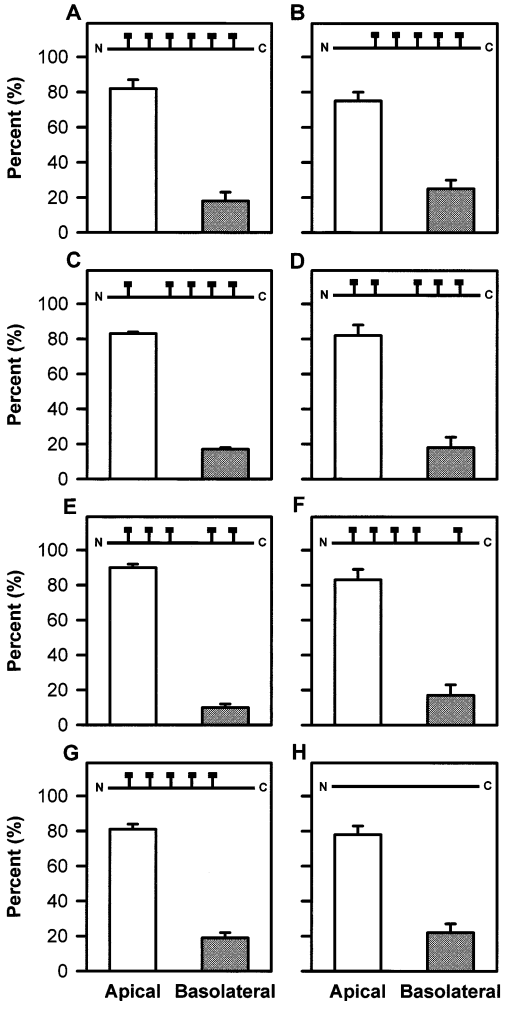

Fig. 4. Mutated and wild-type CBG is secreted mainly to the apical side of MDCK cells. MDCK cells expressing wild-type CBG (A) or CBG mutated at one (B–G) or all (H) *N*-glycosylation sites were grown to confluence on filters, labelled with [^{35}S]methionine and chased for 4 h. The media were collected individually from the apical (open bars) and basolateral (solid bars) side and quantitated via immunoprecipitation (see Fig. 3). The amount of apical or basolateral secretion is shown as percentage of the total secreted CBG (the CBG secreted apical plus the CBG secreted basolaterally). The inserts show a cartoon of the *N*-glycosylation sites present. Mean ± S.D. is shown.

were obtained with a high- and a low-expressing clone. However, the mutant lacking all six *N*-glycosylation sites was expressed at a reduced level compared to the wild-type CBG (roughly estimated to 20% of the wild-type expression) as has also been observed in CHO cells [12]. The DNA sequence of the mutant in which all six *N*-glycosylation sites were eliminated was re-confirmed by DNA sequencing.

It has previously been observed that treatment of transfected MDCK cells expressing CBG with tunicamycin leads to a reduction in the secretion rate of CBG and causes the secretion to change from mainly apical to non-polarized [3]. This could be due to *N*-glycans functioning as the apical targeting signals for CBG [8]. However, as the CBG carrying mutations in all six *N*-glycosylation sites is secreted to the apical side in a manner indistinguishable from wild-type CBG (this study), this is not the case. To investigate the effect of tunicamycin on the secretion of CBG carrying a mutation at all six *N*-glycosylation sites, clones expressing this mutant or wild-type CBG were grown to confluence on filters. The cells were pre-incubated with tunicamycin (15 μg/ml) for 30 min and then labelled for 20 min with [^{35}S]methionine and chased for 4 h, both in the continuous presence of tunicamycin. Mock-treated cells, expressing wild-type or mutated CBG, secreted CBG mainly apically as expected, whereas in tunicamycin-treated cells, we observed a dramatic reduction of the secretion of both wild-type CBG and CBG devoid of *N*-glycans to a level below the detection limit of our assay (data not shown). Musto [3] has previously examined the secretion of wild-type CBG in the presence of tunicamycin and observed a similar reduction, except that he was able to show that the small amounts of CBG were secreted in a non-polarized manner in the presence of tunicamycin. It is at present unclear why tunicamycin reduces the secretion of both wild-type CBG and CBG devoid of *N*-glycans, but we speculate that a critical component of the specialized apical targeting machinery is *N*-glycosylated or otherwise tunicamycin-sensitive.

Up until recently, it has been unclear whether one or more mechanisms exist for apical targeting of secretory proteins. However, recent data indicate that at least two different mechanisms exist. One group of secretory proteins uses *N*-glycans (possibly carrying a specific modification and/or having a specific location on the polypeptides) as apical targeting signal. An example of this is erythropoietin [2] and also growth hormone engineered to carry two *N*-glycosylation sites [8] seems to use this mechanism. Another group of proteins including CBG (this study) and hepatitis B surface antigen [15] are secreted apically using a different targeting determinant. It was shown that neither tunicamycin treatment nor mutagenesis of its single *N*-glycosylation site affected the apical secretion of hepatitis B surface antigen from MDCK cells. The fact that the secretion of CBG is sensitive to tunicamycin makes this drug unsuitable for discrimination between these two groups.

Our results show that the sorting signal responsible for the apical secretion of CBG from MDCK cells is not related to an *N*-glycan structure on CBG and we are currently looking for apical targeting determinants on this protein, by making chimeras between CBG and a non-sorted homologous protein.

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