Secretory cargo composition affects polarized secretion in MDCK epithelial cells

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Abstract Polarized epithelial cells secrete proteins at either the apical or basolateral cell surface. A number of non-epithelial secretory proteins also exhibit polarized secretion when they are expressed in polarized epithelial cells but it is difficult to predict where foreign proteins will be secreted in epithelial cells. The question is of interest since secretory epithelia are considered as target tissues for gene therapy protocols that aim to express therapeutic secretory proteins. In the parathyroid gland, parathyroid hormone is processed by furin and co-stored with chromogranin A in secretory granules. To test the secretion of these proteins in epithelial cells, they were expressed in MDCK cells. Chromogranin A and a secreted form of furin were secreted apically while parathyroid hormone was secreted 60% basolaterally. However, in the presence of chromogranin A, the secretion of parathyroid hormone was 65% apical, suggesting that chromogranin can act as a "sorting escort" (sorting chaperone) for parathyroid hormone. Conversely, apically secreted furin did not affect the sorting of parathyroid hormone. The apical secretion of chromogranin A was dependent on cholesterol, suggesting that this protein uses an established cellular sorting mechanism for apical secretion. However, this sorting does not involve the N-terminal membrane-binding domain of chromogranin A. These results suggest that foreign secretory proteins can be used as "sorting escorts" to direct secretory proteins to the apical secretory pathway without altering the primary structure of the secreted protein. Such a system may be of use in the targeted expression of secretory proteins from epithelial cells.

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Abbreviations

CgA	Chromogranin A
GH	Growth hormone
MDCK	Madin–Darby kidney epithelial
PTH	Parathyroid hormone

Introduction

Polarized secretion is a hallmark of absorptive and secretory epithelia throughout the body. In some cases, including the exocrine glands, apical secretion is a major cellular function while other epithelia primarily secrete proteins to regulate the cellular environment or for host defense. Polarized epithelial cells secrete proteins at either the apical or basolateral cell surface. Multiple cellular

mechanisms have been implicated in either apical or basolateral sorting of secretory and membrane proteins [1, 2]. These include cholesterol rafts, glycosylation, and the pH of the secretory pathway. Endogenous secretory proteins are equipped with the sorting signals necessary for correct targeting to the apical or basolateral cell surface. However, a number of non-epithelial secretory proteins also exhibit polarized secretion when they are expressed in polarized epithelial cells but it is less clear how these proteins are sorted in epithelial cells. Thus, it is difficult to predict where foreign proteins will be secreted in epithelial cells [3]. The question is of interest since secretory epithelia are considered as target tissues for gene therapy protocols that aim to express therapeutic secretory proteins, e.g., peptide hormones, either on the mucosal surface (apical secretion) or in the circulation (basolateral secretion) [4–7]. As an example, hGH expressed in salivary gland epithelial cells is mainly secreted at the apical surface into the oral cavity [8]. To achieve biological effect of the transgene, however, the protein must be redirected to the basolateral pathway for endocrine secretion. In the salivary gland, this can be achieved by in vivo treatment with the weak base hydroxychloroquine [9]. A better understanding of sorting pathways in polarized secretory cells is needed to fully take advantage of epithelial cells for these protein delivery protocols.

A number of sorting mechanisms for secretory proteins have been described in polarized epithelial cells [1]. These include cellular proteins, including galectins 3 and 4 [10, 11], MAL/VIP17 [12–14], SNAP-23 [15], annexin II [16], and α-kinase 1 [17]. In addition specific sorting signals have been identified on individual secretory proteins. Cholesterol-rich rafts have been implicated in the apical secretory pathway in polarized epithelial cells [18, 19]. Reduction of cholesterol levels broadly decreases apical secretion and increases basolateral secretion, suggesting that cholesterol plays a role in the apical transport pathway rather than in the function of specific sorting signals [20]. Apical transport of membrane proteins [21] and secretory proteins, including GP-80 and thyroglobulin [21, 22] can be mediated by cholesterol-rich rafts. Nevertheless, raft association is not a universal sorting mechanism since it is not required for apical secretion of modified GFP [23] or serpins [24] in Madin–Darby Canine Kidney (MDCK) cells.

N-glycosylation has been reported to play a role for apical secretion of several proteins [25]. Upon N-glycosylation, erythropoietin [26], and human growth hormone (hGH) (engineered to include N-glycosylation sites) are secreted apically in MDCK cells [27], but glycosylated hGH is not secreted apically in ECV304 cells [28]. Indeed, N-glycosylation is not necessary for all apical secretion in MDCK cells since corticosteroid binding globulin and osteopontin reach the apical cell surface without N-glycosylation [29, 30]. To determine if cellular N-glycosylation, as opposed to glycosylation of secretory proteins, could play a role in apical sorting, we tested the secretion of chromogranin A, which is not N-glycosylated. Chromogranin A (CgA) is secreted apically in MDCK cells but redirected to the basolateral cell surface when N-glycosylation is inhibited by tunicamycin [31]. O-glycosylation can also play a role in polarized secretion since a domain rich in O-glycosylated Ser and Thr residues is necessary for the apical transport of both membrane-bound and secretory forms of p75 neurotrophin receptor [32]. Mucin-like domains, but not O-glycosylation in general, were sufficient to target secreted green-fluorescent protein to the apical secretory pathway [23]. However, as is the case for N-glycosylation, O-glycosylation is not necessary for apical delivery of all proteins [33].

The mechanisms for basolateral sorting of secretory proteins is less well studied but the pH of secretory organelles appears to play a role since weak bases inhibit basolateral secretion and increase apical secretion in MDCK cells [31, 34]. In this case, modulation of the luminal milieu could serve to modulate the sorting of secretory proteins without altering the structure of the sorted protein. We have recently found that protein sorting can also be modified by manipulating the relative amounts of acidic and basic proteins, either by inhibiting the production of acidic proteins [35] or by overexpressing proline-rich proteins (mainly basic) in chronically isoproterenol-stimulated rats [36]. These findings indicate that the composition of cargo proteins in epithelial cells, could affect the polarity of protein secretion. This is of particular interest when the sorted protein is to serve a therapeutic role after secretion. To further determine how polarized secretion can be modulated without altering the structure or function of the sorted protein, we considered the role of secretory cargo composition on polarized secretion in MDCK cells. The results show that cargo protein composition is sufficient to modulate the sorting of secretory proteins in MDCK cells.

Materials and methods

Materials

Antiserum to GP-80 was from Dr. David D. Sabatini and kindly provided by Dr. Michael J. Rindler, New York University. MDCK cells were from American Type Culture Collection, Manassas, VA. Fetal bovine serum was obtained from Hyclone, Logan, UT while DMEM, Lipofectamine, penicillin/streptomycin and Geneticin were from Life Technologies, Gaithersburg, MD. The expression vector

pcDNA3 was purchased from Invitrogen, Carlsbad, CA. Protease inhibitors and horse-radish peroxidase-conjugated sheep anti-rabbit IgG were from Roche Molecular Biochemicals, Indianapolis, IN. Chemiluminescent peroxidase substrate was from Pierce, Rockford, IL.

Plasmids and stable transfection of MDCK cells

Recombinant DNA techniques were carried out according to standard procedures. A secreted form of mouse furin (s-furin) was provided to us by Dr. R. Mackin, Creighton University who truncated the protein at residue 705, thus eliminating the transmembrane and cytosolic domains. The furin cDNA was originally from Dr. K. Nakayama, University of Tsukuba, Japan. The cDNAs for bovine chromogranin A (bCgA) [37] and s-furin were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). The cDNA for human prepro-parathyroid hormone (preproPTH) (a kind gift of Dr. Henry Kronenberg, Harvard University) was subcloned into pcDNA3.1/zeo(+). MDCK cells were transfected with pcDNA3-bCgA, pcDNA3-s-furin, or with pcDNA3.1-prepro-hPTH using LipofectAMINE (Invitrogen). Beginning 48 h after transfection, multiclonal stable transfectants containing pcDNA3 were selected with 800 µg/ml Geneticin (Invitrogen) while transfectants containing pcDNA3.1-Zeo were selected with 200 µg/ml zeocin (Invitrogen). In double transfection experiments, MDCK/hPTH cells were re-transfected with pcDNA3-bCgA or with pcDNA3-s-furin using LipofectAMINE and selection of multiclonal double stable transfected cells was performed with 800 µg/ml G418 and 200 µg/ml zeocin.

Construction of CgA mutants

Construction of the pcDNA3-CgAwt and pcDNA3-CgAΔcc plasmids was as described [38]. CgA lacking both the N-terminal hydrophobic peak [39] and containing the mutation Cys38Ser (CgAΔNHP) was prepared by PCR based mutagenesis using separate primers (5′-CGTGGTACCTT TATTCATGGGGCTGTT-3′ and 5′-TACGGTACCCCCA TGCCAGTCAGCAAGGAGTCTTTTGAG-3′). Primers for the 3′ and 5′ un-translated regions were as described [38]. The PCR products were joined at a newly created Kpn I site and cloned into pcDNA3. Introduction of the Kpn I site created a conservative point mutation in the CgA coding sequence, Ser30Thr. The identities of the CgA mutants were confirmed by DNA sequencing.

Cell culture and secretion experiments

MDCK cells were cultured and transfected as described earlier [31]. Transfected cells were selected with Geneticin, and colonies were isolated and expanded as described [40]. The cell clones used in this study were maintained in complete medium supplemented with 800 µg/ml of Geneticin.

Secretion experiments were performed as described [31]. Sodium butyrate (5 mM) was used to induce protein expression from the plasmid CMV promoter [41]. This treatment did not affect polarized secretion (not shown). The apical and basolateral secretion media were supplemented with protease inhibitors (1 mM PMSF, 5 mM EDTA) or Complete protease inhibitor cocktail (Roche Molecular Biologicals, Indianapolis, IN) and analyzed by SDS-PAGE and immunoblotting as described [31].

For cholesterol depletion [20, 21], the cells were cultured overnight in complete medium supplemented with 4 µM fluvastatin, and 0.25 mM mevalonic acid lactone. Prior to the secretion experiments, the cells were treated with 10 mM methyl-ß-cyclodextrin for 60 min in complete medium. Cyclodextrin was also included in subsequent wash and secretion buffers.

Floatation gradients

Confluent monolayers of MDCK wt cells were rinsed with PBS and lyzed for 20 min on ice in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (2 ml/10 cm dish). The cells were scraped and homogenized by passage through a 26G needle fitted on a tuberculin syringe. The homogenate was centrifuged for 10 min at 500g to remove cellular debris and the supernatants were adjusted to 40% sucrose. The samples were placed in centrifuge tubes and overlaid with a 5–30% sucrose step gradient and centrifuged at 4°C for 22 h at 200,000g (39,000 rpm, Beckman SW40 rotor). Gradient fractions (1 ml) were collected from the top and their refractive index determined and converted to percent sucrose. The samples were concentrated by trichloro-acetic acid precipitation and analyzed by immunoblotting [31] using a monoclonal antibody to caveolin-1 (BD Transduction Laboratories, Lexington, KY) diluted 1:10,000 in Tris-buffered saline.

RIA for hPTH

The RIA for hPTH was conducted using the Allegro intact hPTH kit (Nichols institute, San Juan Capistrano, CA). Aliquots (200 µl) of secretion media or cell extracts were

incubated with anti-hPTH(39-84)-coated beads and ^{125}I-labeled anti-hPTH(1-34) to form a "sandwich" complex. After washing, the radioactivity bound to the beads was quantitated in a gamma counter.

Immunoblotting for chromogranin A and furin

Aliquots (500 μl) of secretion media or cell extracts were trichloro-acetic acid/acetone precipitated as described earlier [38]. The final pellets were dissolved in SDS-sample buffer and analyzed by SDS-PAGE [42] on 10% gels and transferred to PVDF membranes [43]. Membranes were blocked with 2% Tween-20 in Tris-buffered saline and incubated with either bCgA antiserum (r-Gil #5, generated in our lab) at a dilution of 1:10,000, or monoclonal antibody to mouse furin (MON148 [44]) at a dilution of 1:500 in 0.005%Tween in Tris-buffered saline containing 10 mg/ml BSA. After washing, the blots were incubated with horseradish peroxidase conjugated anti-rabbit (CgA) or anti-mouse (furin) IgG and detected with chemiluminescent substrate [45].

Statistical analysis

Where appropriate, the data are expressed as mean ± s.e.m. Control and treated samples were analyzed by Student's t-test and $P < 0.05$ was considered statistically significant.

Results

As a model of polarized secretion of peptide hormone in epithelial cells, we expressed PTH in MDCK cells. PTH is normally expressed in parathyroid chief cells where it is stored in secretory granules and secreted to the circulation in response to low plasma calcium concentrations [46]. Unlike endocrine or exocrine glands, MDCK cells do not contain secretory granules and, therefore, allow the analysis of polarized sorting without any contribution from a regulated secretory pathway. In MDCK cells, PTH was secreted predominantly in an endocrine fashion (basolateral secretion) (Fig. 1a) and this polarized secretion of PTH was consistent over a broad range of PTH expression (Fig. 1b).

To test if apical secretory proteins could redirect PTH to a more apical secretion, we co-expressed PTH with two different apical secretory proteins. We have previously reported that the endocrine protein CgA is secreted by the apical pathway in MDCK cells [31]. As a further test protein, we expressed a secreted form of furin in MDCK
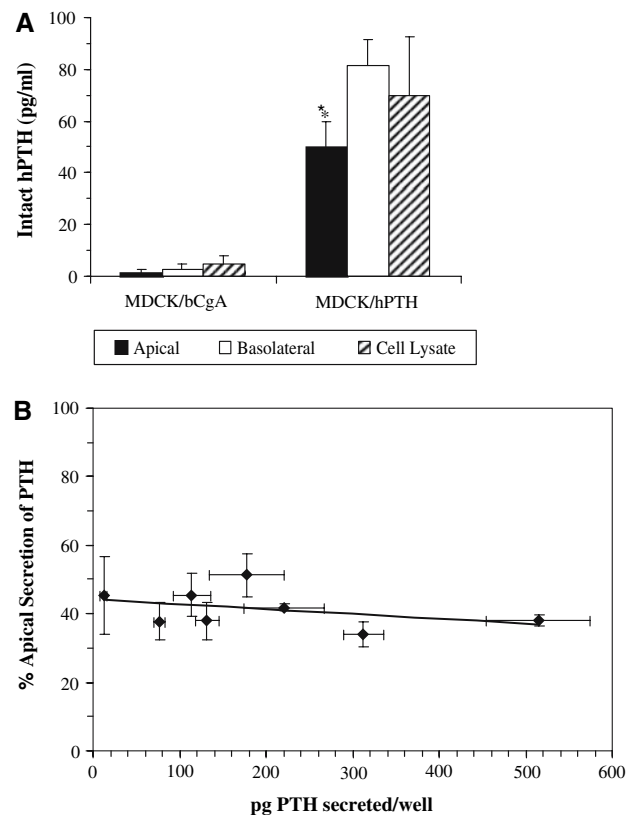
Fig. 1 Expression of hPTH in MDCK cells. (**a**) Filter-grown MDCK cells stably expressing hPTH (MDCK/hPTH) or bCgA (MDCK/bCgA) were analyzed for the distribution of apical secretion (filled bars), basolateral secretion (open bars) and cell lysate content (hatched bars) of hPTH. Cells were cultured in the presence of 5 mM Na-Butyrate for 18–20 h prior to secretion experiments. Intact hPTH in the samples was analyzed by RIA and expressed as pg/ml (Different from basolateral secretion, *$P < 0.02$, $n = 3$). (**b**) The % apical secretion (apical secretion/apical + basolateral secretion) × 100% was plotted vs. the mean of the total amount of secreted PTH in each culture well (pg PTH secreted/well). Each data point represents the mean ± SD of a single experiment, $n = 3$–6

cells. Furin is responsible for proteolytic processing of proPTH in the parathyroid gland, suggesting that the two proteins can interact in the secretory pathway. The wild-type membrane bound form of furin is expressed at both the apical and basolateral plasma membrane in MDCK cells, with a preference for the latter [47] consistent with the presence of a basolateral sorting signal in the membrane domain [48]. Accordingly, when truncated furin, lacking the transmembrane and cytosolic domains, was expressed in MDCK cells, the protein was secreted exclusively from the apical side of the cells (Fig. 2a). To test if the apical secretion of furin could affect the polarized secretion of PTH, the peptide hormone was co-expressed with s-furin. Figure 2b shows that overexpression of s-furin did not alter the polarized secretion of PTH, suggesting that the two proteins are not strongly associated in the secretory pathway.

Furin Western Blotting

A

Intact hPTH Immunossay

B
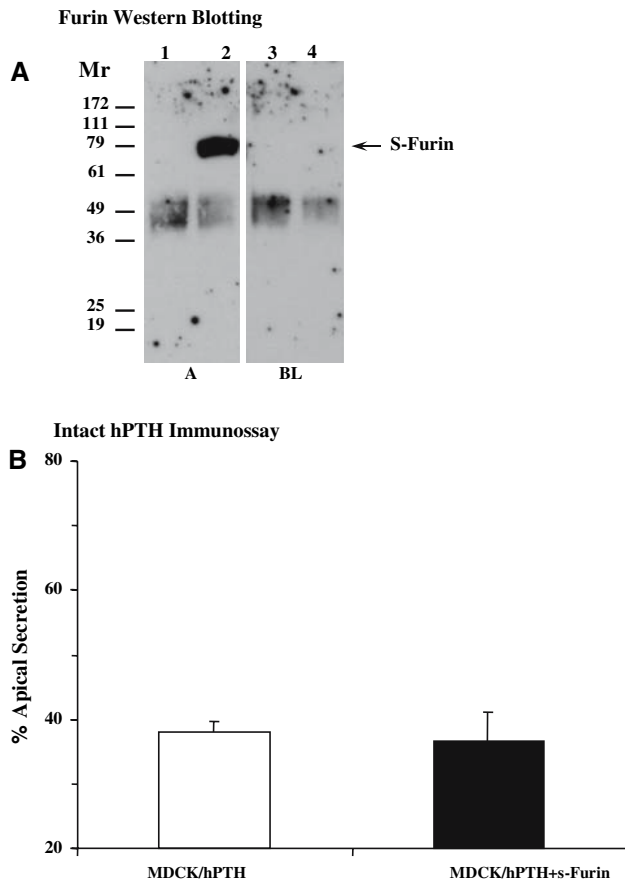
Fig. 2 Effect of furin on polarized secretion of hPTH. Filter-grown MDCK/hPTH or MDCK/hPTH+s-furin cells were analyzed for the distribution of apical and basolateral secretion of truncated secreted furin (s-furin) (**a**) and hPTH (**b**). (**a**) Secreted furin was analyzed by western blotting. Lanes 1 and 3: MDCK/hPTH (negative control); lanes 2 and 4: MDCK/hPTH+s-furin. Each lane shown is representative of six samples. The experiment was repeated with similar results. (**b**) Intact hPTH in the samples was analyzed by RIA. % apical secretion = (apical secretion/apical + basolateral secretion) × 100%. Data are presented as the mean ± s.e.m. ($n = 6$). The experiment was repeated with similar results

PTH is a polyanion binding protein [49] and we have previously found that CgA, an acidic sulfated glycoprotein, co-aggregates with PTH under the conditions that exist in the trans-Golgi network [50]. To test if CgA could affect PTH secretion, the proteins were co-expressed in MDCK cells. Figure 3 shows that CgA significantly increased the apical secretion of PTH from 38% apical in the absence of CgA to 65% apical in the presence of CgA. The sequence of transfection was not important since the effect was seen in two different stable cell lines (see Methods and methods for details).

It has been reported that entry into the apical secretory pathway can be saturated in polarized RPE-J cells [51]. To test if overexpression of CgA affected apical secretion in general, we analyzed the apical secretion of the endogenous
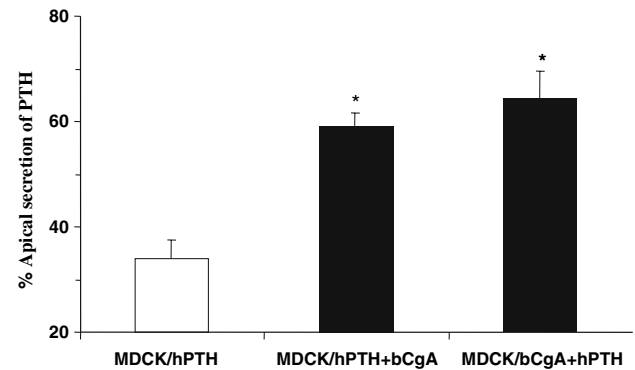
Fig. 3 CgA-induced apical delivery of hPTH in MDCK cells. Filter-grown MDCK/hPTH, MDCK/hPTH+bCgA or MDCK/bCgA+hPTH cells were analyzed for the distribution of apical and basolateral secretion of hPTH by RIA. % apical secretion = (apical secretion/apical + basolateral secretion) × 100%. The data are presented as the mean ± s.e.m. ($n = 6$). *Different from MDCK/hPTH, $P < 0.0001$

apical secretory protein GP-80 in the presence or absence of CgA. In the absence of CgA, secretion of GP-80 was $79 \pm 1.0\%$ (mean ± s.e.m. $n = 4$) apical, consistent with a previous report [52] compared to 77% apical secretion of GP-80 in MDCK cells that express CgA [31]. As a further control, we noted that secretion of the apical marker protein SEAP [31] was 75–80% apical over an expression-level range of two orders of magnitude in MDCK cells (not shown).

It is thought that apical secretory proteins are sorted by interaction with membrane components of MDCK cells [2], including cholesterol-rich membrane rafts [20, 21]. To confirm the presence of cholesterol rafts in our cultured MDCK cells, a Triton X-100 detergent solubilized cell homogenate was subjected to density gradient centrifugation. The gradient fractions exhibited peaks in cholesterol (not shown) and caveolin 1, a marker for cholesterol rafts (Fig. 4a) in fractions containing 23–25% sucrose. This is consistent with the presence of cholesterol rafts in MDCK cells. To test if cholesterol played a role in CgA sorting, MDCK cells were treated with fluvastatin, mevalonate, and methyl-ß-cyclodextrin to reduce cellular cholesterol synthesis and content. The secretion of CgA and the endogenous apical secretory protein GP-80 were analyzed by immunoblotting (Fig. 4b). Both proteins were secreted apically in untreated cells. However, after cholesterol depletion, GP-80 was secreted randomly at the apical and basolateral surface (50% each) (Fig. 4c) while CgA was secreted predominantly (71%) from the basolateral cell surface (Fig. 4c). Thus, cholesterol is required for apical sorting of both CgA and GP-80. The difference in GP-80 and CgA secretion in fluvastatin-treated cells suggests that the proteins are actively secreted rather than passively diffusing, e.g., by a paracellular pathway.
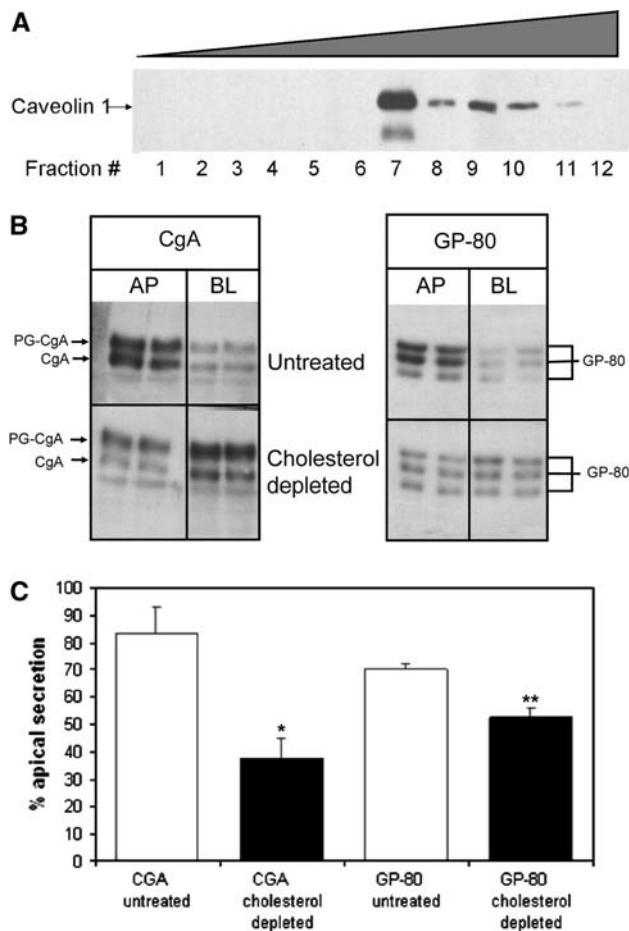
Fig. 4 Role of cholesterol in apical secretion of CgA. (**a**) Identification of cholesterol rafts. MDCK cell homogenates were separated by floatation in a 5–30% sucrose step gradient. Fractions were collected from the top and analyzed for caveolin-1 contents by immunoblotting. Fraction numbers and the position of caveolin-1 are indicated. (**b**) Cholesterol depletion. Secretion of CgA and GP-80 was analyzed in untreated and cholesterol-depleted MDCK cells. Apical (AP) and basolateral (BL) secretion media were analyzed by immunoblotting. The positions of CgA, proteoglycan CgA and GP-80 are indicated. (**c**) Quantitation of the Western blot results by densitometric scanning. % apical secretion = (apical secretion/apical + basolateral secretion) × 100%. The data are presented as mean ± s.e.m., $n = 3$–8. *Different from untreated control, $P < 0.01$, **Different from untreated control $P < 0.0001$

The mechanism for cholesterol-dependent apical sorting of CgA is not known but the N-terminal domain of CgA has been implicated in membrane-binding of this protein [53]. This domain consists of an N-terminal hydrophobic domain [39] that is part of a disulfide bonded loop, which is necessary for sorting of chromogranins to secretory granules in PC12 cells [38]. To test if the N-terminal hydrophobic domain or the N-terminal disulfide loop were necessary for apical sorting of CgA, the Cys residues forming the N-terminal disulfide loop and the entire N-terminal domain (including Cys residues) were deleted
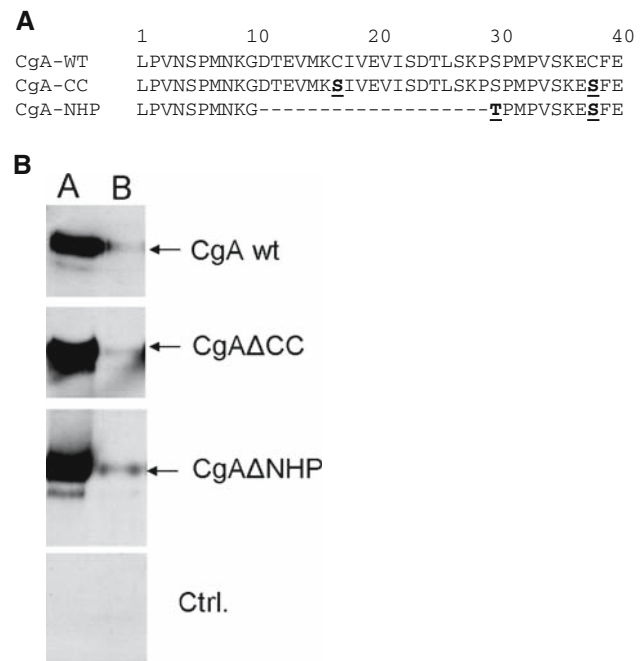
Fig. 5 Expression of CgA mutants in MDCK cells. (**a**) N-terminal Sequences of the CgA mutants (without signal peptides). (**b**) CgA mutants were expressed in MDCK cells and the apical (A) and basolateral (B) secretion media were analyzed by immunoblotting. Each condition is representative of triplicate samples from a single experiment

in separate CgA mutants (Fig. 5a). The secretion of wild-type and mutant forms of CgA was tested in polarized MDCK cells. Figure 5b shows that both mutants exhibited apical secretion that was similar to the secretion of wild-type CgA. This indicates that the N-terminal membrane-binding domain is not required for apical sorting of CgA.

Discussion

Polarized epithelia are attractive targets for gene therapy protocols since they are often readily accessible (e.g., in the lungs and GI tract) and the vector need not be introduced systemically to achieve expression. However, to achieve therapeutic effect, the transgene products must be correctly targeted to either the mucosal surface (apical secretion) or circulation (basolateral secretion). Indeed, foreign secretory proteins can be sorted in polarized epithelial cells, although the direction of their secretion is not directly predictable. As an example, secretion of hGH is apical in salivary epithelial cells [8], non-polarized in MDCK cells [54], and basolateral in colon epithelial Caco-2 cells [55]. The endocrine secretory granule protein CgA is sorted to the apical cell surface in polarized MDCK cells [31] while PTH, which is co-stored with CgA in secretory granules of endocrine cells, is mainly secreted at the basolateral cell

surface (this report). In each case, the secretory protein presumably interacts with the cellular sorting machinery for the apical and basolateral secretory pathway respectively. In this study, we asked if such interactions could be exploited to redirect protein secretion by co-expressing different secretory proteins. As a model experimental system, we co-expressed PTH in MDCK cells with the apical secretory proteins s-furin and CgA, which have been implicated in proPTH processing and storage, respectively. CgA was able to partially redirect PTH to the apical secretory pathway. Indeed, the 65% apical secretion of PTH obtained in cells that co-express CgA is close to the apical secretion of the endogenous secretory protein GP-80 (about 80% apical). If we assume that this represents the maximum efficiency of apical secretion in MDCK cells, CgA re-directed PTH with over 80% efficiency. These results suggest that "protein escorts" (sorting chaperones) can be used to redirect the secretion of a peptide hormone in polarized epithelial cells.

Unlike membrane-bound furin, which is transported with a polarity similar to that of PTH secretion [47], secreted furin is released at the apical cell surface (Fig. 2a). The apically secreted furin was unable to redirect PTH, although PTH is a substrate for furin processing in the secretory pathway [56]. Since furin converts proPTH to PTH [56], it is possible that the mature PTH peptide is released by furin shortly after processing of the precursor. Although, we found no rate-limiting effects of the level of protein expression in the apical or basolateral pathways in MDCK cells, we cannot completely rule out that the expression level of furin was insufficient to affect PTH secretion.

In addition to the modulation of PTH secretion by protein escorts, this report shows that apical secretion of CgA depends on cellular cholesterol. A similar conclusion has been reported for the polarized secretion of erythropoietin in MDCK cells [57] whereas apical secretion of serpins is independent of raft association [24]. This difference in use of cellular sorting mechanisms has also been noted for cellular N-glycosylation, which is necessary for apical sorting of CgA in MDCK cells [31], but not for sorting of an unglycosylated form of hepatitis B surface antigen [58].

Together the above results confirm that the secretion of foreign proteins from MDCK cells can be modulated by manipulating the cellular milieu without changing the structure of the secreted protein. This is desirable from an in vivo application standpoint, since altering the structure of the secreted protein could also alter its function and/or stability in vivo. On the other hand, changing the cellular milieu with inhibitors of cholesterol or oligosaccharide synthesis, are broad measures that affect many cellular functions and are not specific for the cells that express the protein of interest. The use of "sorting escorts" may offer an alternate approach for the targeted delivery of therapeutic secretory proteins from transfected epithelial cells. Since the protein of interest and the "sorting escort" can be co-expressed in individual cells, only these cells would be affected by the changes in protein secretion. Other cells in the tissue or in other tissues would be unaffected. Together with our recent reports on aggregation chaperones that can modulate the storage of secretory proteins in endocrine cells [59, 60], these results suggest that the protein composition in the secretory pathway can be modified to regulate protein transport.

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