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Biochemical and Biophysical Research Communications 299 (2002) 35–41
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Serpins are apically secreted from MDCK cells independently of their raft association

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Received 8 October 2002

Abstract

It has been suggested that detergent-resistant membranes (DRMs), also known as lipid rafts, are involved in vectorial transport of proteins to the apical surface. In this report we use Madin–Darby canine kidney (MDCK) cells expressing the apically secreted C1-esterase inhibitor, the non-sorted antithrombin or chimeras of serpins to study the possible connection between DRM association and apical targeting of secretory proteins. We found newly synthesised C1-esterase inhibitor associated with DRMs in MDCK cells, whereas antithrombin was not. However, two chimeric proteins, secreted mainly from the apical membrane, do not associate with DRMs. Based on these observations we suggest that apical targeting and association with DRMs are two independent events for secretory serpins.

Keywords: Madin–Darby canine kidney cells; Rafts; Serpins; Apical targeting

The epithelial plasma membrane is spatially separated by tight junctions into an apical and a basolateral domain which differs in lipid and protein composition [1,2]. For the establishment and maintenance of this specific composition, intracellular sorting machineries in the epithelial cells ensure correct targeting of both proteins and lipids to the two domains. In Madin–Darby canine kidney (MDCK) cells sorting of newly synthesised proteins destined for the apical or basolateral surfaces is thought to occur in the trans-Golgi network (TGN) [3,4].

Basolateral targeting of transmembrane proteins is mediated by sorting signals in their cytoplasmic domain that might or might not be related to dileucine- or tyrosine-based sorting signals [4,5]. Much less is known about the signals and mechanisms involved in apical targeting. Apical sorting signals on trans-membrane proteins appear to be very heterogeneous as they in some cases have been located to the extracellular domain [6–8], in some cases to the trans-membrane domain [9–11], and in other cases to the cytoplasmic tail [12,13]. For secretory proteins it has been shown that *N*-glycans may act as an apical sorting signal [14]. However, N-linked glycosylation is not the only apical targeting signal for secretory proteins, since mutagenesis of the 6 N-glycosylation sites of corticosteroid binding globulin (CBG), individually or together, did not affect its apical secretion from MDCK cells [15]. N-glycosylation has also been shown to be of no importance for apical targeting of the secreted Hepatitis B surface antigen [16] and a soluble form of the p75 neurotrophin receptor [17]. Thus, there seem to be at least two mechanisms of apical sorting of secretory proteins, either dependent or independent of *N*-glycans. We have focused on *N*-glycan independent apical targeting, using members of the serpin family as model proteins. Some of them, including CBG, α1-antitrypsin (α1), and C1 inhibitor (C1), were found to be secreted apically from MDCK cells, whereas other members, including antithrombin (AT) and plasminogen activator inhibitor-1 (PAI-1) [18], are secreted in a non-sorted manner. The structural similarity allowed the construction of chimeras to identify the sequence requirements for apical sorting and showed

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PII: S0006-291X(02)02577-9

that amino terminal segments of the three serpins CBG, α1, and C1 are sufficient to direct the normally non-sorted AT to the apical side of MDCK cells [19].

An apical sorting mechanism has been suggested to involve specific incorporation into distinct membrane microdomains, called detergent-resistant membranes (DRMs) or rafts. The existence of DRMs in living cells has been heavily debated, but two recent reports showed that GPI-anchored proteins are clustered in cholesterol-containing membrane microdomains at the cell surface of both MDCK cells and CHO cells [20,21]. DRMs are formed in the Golgi apparatus and are enriched in cholesterol and glycosphingolipids and it has been proposed that they function as sorting platforms for apical targeting of secretory and trans-membrane proteins, respectively [22]. Several proteins associated with DRMs are known to be important for apical protein transport (e.g., caveolin/VIP21 [23], annexin XIIIb [24,25], the apical v-SNARE TI-VAMP [26], and the proteolipid MAL [27,28]). Depletion of endogenous MAL in MDCK cells by antisense RNA prevents apical transport of both membrane- and secretory proteins [27–30], suggesting a connection between apical targeting and DRM association.

Secretory proteins are cotranslationally delivered to the lumen of the rough endoplasmic reticulum, and hence, in contrast to membrane-bound proteins, they remain luminal throughout their passage through the biosynthetic pathway. Accordingly, association between secretory proteins and membrane microdomains is indirect and mediated through a membrane-bound component. The first example of this type of raft association was recently shown for the apically secreted protein thyroglobulin [31]. In contrast to thyroglobulin, clusterin/gp80, the major apically secreted endogenous protein of MDCK cells, is not DRM associated during biosynthetic transport in MDCK cells [32], despite the fact that normal apical targeting of gp80 requires intact DRMs [33].

In the present paper we used wild type serpins (C1 and AT) and three apically directed chimeras, each containing the amino terminal segments of one of the three apically secreted serpins, CBG, α1, and C1, to study whether a correlation between apical secretion and DRM association exists. We found the apically sorted C1 associated with DRMs whereas no association could be observed for the non-sorted AT. However, only one of the three apically secreted chimeras was DRM associated demonstrates that apical transport can occur without association with DRMs.

Materials and methods

Materials. Expression vectors pTEJ-4 containing the cDNA coding for rat AT and human C1 were previously described [18]. The cDNAs coding for chimeras $CBG_{1–30}AT$, $\alpha1_{1–39}AT$, and $C1_{1–132}AT$ are described elsewhere [19].

Antibodies used were a sheep polyclonal antibody against human C1 (Quidel, San Diego, CA, USA), a rabbit polyclonal antibody against human C1 (DAKO, Copenhagen, Denmark), an affinity-purified sheep polyclonal antibody against rabbit AT (a gift from Dr. W.P. Sheffield, McMaster University, Canada), a rabbit polyclonal antibody against caveolin-1 (Transduction Laboratories, Lexington, KY, USA), and a peroxidase-conjugated secondary anti-IgG antibody (DAKO, Copenhagen, Denmark). Protein A–Sepharose CL-4B was obtained from Pharmacia Biotech (Uppsala, Sweden); Complete protease-inhibitor cocktail was from Roche Diagnostics GmbH (Mannheim, Germany); Triton X-100 and methyl-β-cyclodextrin (m-β-CD) were from Sigma Chemical (St. Louis, MO, USA); [^{35}S]methionine was from Dupharma (Copenhagen, Denmark); and lovastatin was a gift from Merck Sharp and Dohme (Rahway, NJ, USA).

Cell culture and transfection. MDCK cells (strain II), a gift from Dr. K. Mostov, University of California, were grown in Minimum Essential Medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal calf serum, penicillin (50 U/ml), and streptomycin (50 mg/ml), at 37 °C in an atmosphere of 5% CO_2. CHO-K1 cells, a gift from Dr. B. van Deurs, University of Copenhagen, Denmark, were grown in Ham's F12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% foetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml). For detergent-extraction experiments, cells were grown in tissue culture flask (150 cm^2; Techno Plastic Products AG, Switzerland) and used for experiments 2–3 days after confluency. This should ensure full differentiation of the MDCK cells [1]. The TGN-to-surface transport was analysed after seeding 10^6 cells onto Transwell filters (No. 3412; Costar Europe, Badhoevedorp, the Netherlands). Cells were used for sorting experiments 2–3 days after confluency as indicated by assessing the tightness of the filter [34] and by measuring the transepithelial resistance (TER) using a Millicell ERS voltohmmeter (Millipore Continental Water System, Bedford, MA, USA). MDCK cells were transfected and selected as previously described [15,18]. CHO cells (10^5) were transfected using the same transfection protocol as for MDCK cells. Beginning at 48 h post-transfection stable transformants were selected by treatment with 0.65 mg/ml G418. Resistant CHO-K1 cells were pooled and subsequently used for experiments.

Depletion of intracellular cholesterol. To deplete cells for cholesterol the protocol described by Keller and Simons [33] was used. Briefly, confluent MDCK cells on filters were incubated with 4 μM lovastatin for 48 h at 37 °C. Filters were only used if a TER of >450 Ωcm^2 was maintained throughout the experiment. The cells were then treated for 30 min with 10 mM methyl-β-cyclodextrin (m-β-CD) in medium containing lovastatin (750 μl apically; 1500 μl basolaterally). This treatment reduced the amount of intracellular cholesterol with ~60–70% as previously described [33,35]. A decrease of this magnitude was recently shown to increase the TER, and only prolonged periods of incubation (>2 h) with m-β-CD affected the permeability of the monolayer [35].

Metabolic-labelling, TGN-to-surface transport, and immunoprecipitation. Secretion of C1 and gp80 was assayed according to a previously published method [33]. Briefly, cells were pulse-labelled for 15 min at 37 °C with ~160 μCi [^{35}S]methionine, chased for 5 min at 37 °C, and further incubated at 19.5 °C for 75 min. Then the cells were shifted to 37 °C for 30 min and the apical and basolateral media were collected. The amount of secreted C1 and AT was determined using quantitative immunoprecipitation as previously described [15,18]. The endogenous gp80/clusterin is the predominant protein secreted from MDCK cells [36]. Accordingly, the amount of gp80/clusterin was detected in the media simply by analysis of culture medium by SDS–PAGE under non-reducing conditions [37]. The amount of ^{35}S-labelled gp80/clusterin was determined using a phosphoimager (Molecular Dynamics PhosphoImager SI). The relative amount of C1 (and gp80/clusterin) secreted apically was calculated: C1 apically secreted (%) = C1 in apical

medium × 100/[C1 in apical medium + C1 in basolateral medium]. The amount of basolaterally secreted C1 was calculated accordingly.

Detergent-extraction procedures and sucrose density centrifugation. Flotation of DRMs by sucrose gradient centrifugation was performed essentially as described [38,39]. Briefly, confluent MDCK cells were scraped from a tissue culture flask (150 cm^2), pelleted in a centrifuge (800*g*; 3 min), resuspended in 25 mM HEPES, 25 mM MES (pH 6.2, unless otherwise indicated), and 150 mM NaCl containing 1% Triton X-100, incubated at 4 °C for 15 min, and then mixed with an equal amount of 80% sucrose in the same buffer. All buffers were supplemented with Complete protease-inhibitor cocktail. Step-gradients were made by laying 6 ml of 30% sucrose-buffer and 2 ml of 5% sucrose-buffer on top of the 40% sucrose cell extract. After centrifugation in a Beckman SW40 Ti rotor (Beckman Instruments, Palo Alto, CA, USA) at 35.000 rpm (g_{max} =217,000) for 18–20 h at 4 °C gradients were fractionated (1 ml/fraction).

Immunoblotting. Total protein was precipitated using acetone (1:1 vol/vol), solubilized in reducing SDS–PAGE buffer and run on 15% SDS–PAGE gels, and semidry blotted to Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% BSA, probed with C1 antibody (1:5000) or AT antibody (1:10,000), probed with appropriate secondary peroxidase-conjugated antibody, incubated with enhanced chemiluminescent substrate (Pierce Chemical, Rockford, IL), and developed with a Fuji LAS1000-camera (FujiFilm Sverige AB, Stockholm, Sweden). The relative amount of DRM associated C1 (fractions 5–12) was calculated as follows: DRM associated C1 (%) = $C1_{Frac.\,5–12} \times 100/[C1_{Frac.\,5–12} + C1_{Frac.\,1–4}]$.

Results

C1 is associated with DRMs, whereas AT is not, during transport to the plasma membrane

To investigate if C1 and AT are DRM associated during their passage through the secretory pathway we examined their solubility in Triton X-100 (pH 6.2) at 4 °C as analysed by sucrose gradient centrifugation. Proteins associated with insoluble DRMs float to low-density fractions in the gradient while other insoluble proteins, such as cytoskeletal proteins and soluble proteins, remain in the high-density fractions [39]. As shown in Fig. 1A, the majority of intracellular C1 remained in the high-density fractions (fractions 1–4). However, some C1 was recovered from the low-density fractions (fractions 5–10) of the gradient showing that a fraction of the proteins is associated with DRMs. We have previously shown that C1 secreted from MDCK cells also occurs as two distinct bands on SDS–PAGE gels [18]. The low molecular form of C1 was recovered more efficiently from DRM fractions compared with the high molecular form (Fig. 1A). This could further suggest a correlation between DRM association and apical targeting as the low molecular form of C1 is more efficiently targeted to the apical membrane in MDCK cells [18]. In contrast to C1, no AT could be recovered from the low-density fractions (Fig. 1B). After initial immunoblotting the PVDF membranes were stripped and re-blotted with anti-caveolin-1 antibody as positive control for flotation of DRMs to low-density fractions in the sucrose gradient (Fig. 1C). Caveolin-1 was predominantly recovered from fractions surrounding the border between 30% and 5% sucrose. This corresponds to the localisation of the visible lipid band in the sucrose gradient. The C1 recovered from low-density fractions devoid of caveolin-1 may represent molecules, which have dissociated from the caveolin-1 enriched DRM fractions during the centrifugation. A similar distribution was recently observed for the secretory protein thyroglobulin [31].

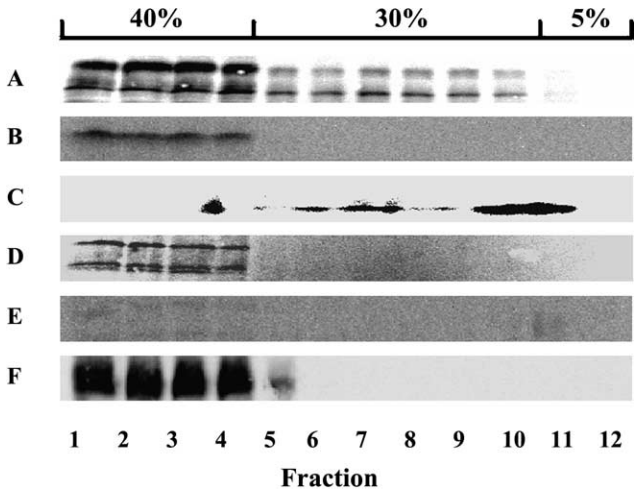

Fig. 1. C1, but not AT, associates with DRMs in MDCK cells. MDCK cells stably expressing recombinant C1 (A, C, D, and E) or AT (B) were extracted with 1% Triton X-100 in 25 mM HEPES; 25 mM MES; and 150 mM NaCl (pH 6.2) at 4 °C, and centrifuged overnight to equilibrium in a sucrose step-gradient. Fraction 1–4 contains the Triton X-100 soluble material, while fraction 5–12 are the lower density fractions containing the Triton X-100 insoluble material. The amounts of C1, AT, and caveolin-1 in each fraction were determined by SDS–PAGE and immunoblotting. (A) Blot probed with antibody against C1. (B) Blot probed with antibody against AT. (C) Blot probed with antibody against caveolin-1, a protein enriched in DRMs. (D) MDCK cells, expressing C1, were treated with 4 μM lovastatin and 10 mM methyl-β-cyclodextrin. Blot probed with antibody against C1. (E) After centrifugation, samples from each fraction were incubated with proteinase K. Blot probed with antibody against C1. (F) CHO cells stably expressing recombinant C1 were extracted with Triton X-100 as described above. The amount of C1 in each fraction was determined by SDS–PAGE and immunoblotting.

The flotation of C1 to low-density fractions was shown to be dependent on cholesterol as culturing the cells in the presence of lovastatin and m-β-CD before extraction abolished the flotation of C1 in the gradient (Fig. 1D). To rule out the possibility that the association of C1 to DRMs was due to unspecific capturing in DRM-enriched vesicles during treatment with 1% Triton X-100 we incubated fractions with proteinase K. As shown in Fig. 1E incubation with proteinase K (200 μg/ml) completely digested all C1 both in low- and high-density fractions of the sucrose gradient. This shows that C1 is accessible to proteinase K, indicating that the association is not due to unspecific capturing in DRM-vesicles during incubation with detergent. To determine

if association of C1 with DRMs was restricted to polarised MDCK cells or the association could be observed in other cells types, C1 was stably expressed in non-polarised fibroblasts (Chinese Hamster Ovary (CHO) cells). In contrast to MDCK cells, C1 was completely soluble in Triton X-100 (Fig. 1F) as it fractionated at the bottom of the sucrose gradient (fractions 1–4).

Depletion of intracellular cholesterol inhibits apical targeting of C1

DRMs are known to be destabilised by cellular cholesterol depletion [40] and in MDCK cells this leads to impaired apical targeting of the endogenously expressed secretory protein, gp80/clusterin [33]. To determine the effect of cholesterol depletion on C1 targeting we treated MDCK cells with lovastatin (4 μM) and m-β-CD (10 mM). As a positive control, we also examined the endogenously expressed gp80/clusterin. Using the TGN-to-surface assay (see Materials and methods) we showed that cholesterol depletion of MDCK cells profoundly affected apical targeting of C1 (Fig. 2A) and gp80/clusterin (Fig. 2B). Both C1 and gp80/clusterin were secreted mainly apically in non-treated cells and in a non-polarised fashion after treatment with lovastatin/m-β-CD.

Treatment with lovastatin/m-β-CD did not influence the TER (data not shown), showing that the integrity of the cell layer was maintained. However, this observation did not exclude the fact that lovastatin/m-β-CD could, somehow, induce transcytotic transport of secreted C1 from the apical to the basolateral medium, and thereby hide an initial biosynthetic targeting to the apical medium. We analysed this possibility by adding ^{35}S-labelled C1 to either the apical or basolateral side of tight filter-grown untransfected MDCK cells treated with lovastatin/m-β-CD. The amount of C1 in the media was determined after 125 min by quantitative immunoprecipitation. No transcytosis from either the apical or basolateral medium could be observed (data not shown). This shows that depletion of intracellular cholesterol affects the biosynthetic targeting of C1 and gp80/clusterin.

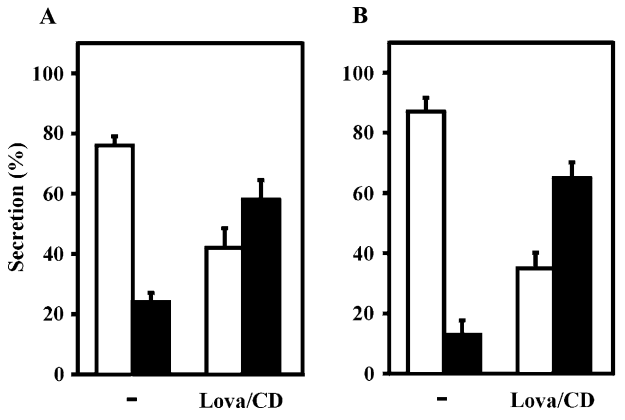

Fig. 2. Apical targeting of C1 is disturbed by cholesterol depletion, but not by inhibition of glycosphingolipid synthesis. MDCK cells expressing C1 were grown on filters until confluence in the presence or absence of 4 μM lovastatin and 10 mM methyl-β-cyclodextrin (Lova/CD). The TGN-to-surface targeting was measured as described (see Materials and methods). Chase media were collected from the apical (open bars) and the basolateral (black bars) media. (A) Biosynthetic targeting of C1. (B) Biosynthetic targeting of gp80/clusterin. The amount of apical or basolateral secretion is shown as a percentage of the total secreted C1 or gp80/clusterin. Means ± SD is shown (C1: $n = 6$; gp80/clusterin: $n = 3$).

Two apically targeted chimeric proteins do not associate with DRMs

It has previously been described that fusion of the amino terminal part of the apically targeted corticosteroid-binding globulin (CBG), α1-antitrypsin (α1) or C1 to the complementary carboxyl terminus portion of the non-polarised secreted AT is sufficient to confer apical targeting ability to the resulting chimeric proteins [19]. The chimer $CBG_{1–30}AT$ consists of residue 1–30 of CBG and the complementary carboxyl terminus portion of AT. The chimeras, $\alpha1_{1–39}AT$ and $C1_{1–132}AT$, consist of residues 1–39 of α1 and 1–132 of C1, respectively, likewise fused to their complementary carboxyl terminus of AT. $CBG_{1–30}AT$ and $\alpha1_{1–39}AT$ are fused in the same point, whereas $C1_{1–132}AT$ are fused one amino acid further towards the amino terminus. $CBG_{1–30}AT$, $\alpha1_{1–39}AT$, and $C1_{1–132}AT$ are secreted mainly apically from MDCK cells [19]. We asked whether a correlation between apical targeting and DRM association could be observed for the three chimeras. This was analysed as previously done by incubation with Triton X-100 in the HEPES/MES buffer (pH 6.2) at 4 °C, sucrose density centrifugation followed by SDS–PAGE and immunoblotting. The chimeras, $CBG_{1–30}AT$, $\alpha1_{1–39}AT$, and $C1_{1–132}AT$, were detected using the same antibody as previously used to detect AT. As shown (Fig. 3B), the majority of $CBG_{1–30}AT$ fusion protein remained in the high-density fractions in the bottom of the gradient; however, a small fraction was observed to float into low-density fractions, showing that the $CBG_{1–30}AT$ fusion protein associates with DRMs. However, analysis of the remaining two fusion proteins, $C1_{1–132}AT$ and $\alpha1_{1–39}AT$, showed that neither of them associates with DRMs (Fig. 3A and C). The presence of DRMs in the light density fractions was confirmed by immunoblotting with antibody against human caveolin-1 (data not shown). This shows that no correlation exists between apical targeting and DRM association of secretory proteins.

pH-dependent association of C1 with DRMs

Association of secretory proteins with DRMs has to be mediated through a membrane bound component.

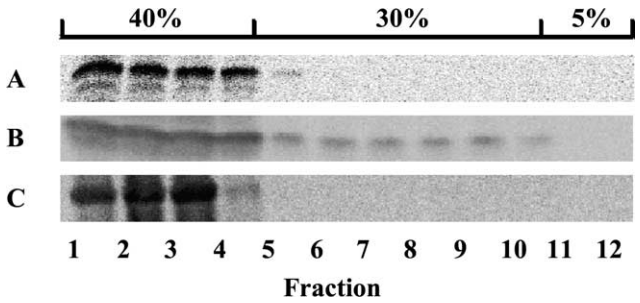

Fig. 3. Only one out of three apically secreted chimeric proteins is DRM associated. MDCK cells stably expressing $C1_{1–132}$AT (A), $CBG_{1–30}$AT (B), and $\alpha1_{1–39}$AT (C) were extracted with 1% Triton X-100 in 25 mM HEPES; 25 mM MES; and 150 mM NaCl (pH 6.2) at 4 °C, and centrifuged overnight to equilibrium in a sucrose step-gradient. The amount of chimeric protein in each fraction was determined by SDS–PAGE and immunoblotting. All blots were probed with antibody against AT.

Such an interaction between a secretory protein and a DRM-associated membrane component could be pH-sensitive. In order to test this we used Triton X-100 extraction buffers at three different pH values. Briefly, cells were prepared for extraction (as described in Materials and methods); however, the Triton X-100 extraction buffer was adjusted to three different pH values (i.e., 5.5, 6.2, and 7.5). After incubation, the cell extract was adjusted to 40% sucrose in the appropriate buffer. After ultracentrifugation overnight fractions were collected from the top of the gradient and the presence of C1 and caveolin-1 in the DRM fractions was analysed by immunoblotting. Using a Fuji LAS-1000 chemiluminescence camera the ratio between insoluble DRM-associated protein (fractions 5–12) and soluble protein in the lower fractions (fractions 1–4) was determined. When the Triton X-100 extraction procedure was performed in buffer with pH 6.2 20% (SD = 2%; $n = 3$) of the intracellular C1 could be recovered in DRMs (Fig. 4 and Fig. 1A). Further pH-decrease in the Triton X-100 buffer to pH 5.5 resulted in essentially the same degree of DRM-association, as 24% (SD = 6; $n = 3$) of intracellular C1 migrated to lighter fractions in sucrose density gradients. In contrast, preparation of DRMs in Triton X-100 buffer, pH 7.5, decreased the association of C1 with DRMs significantly, as only 5% (SD = 3%; $n = 3$) of C1 was recovered from the top fractions of the gradient. To exclude the possibility that pH could have a general effect on DRM flotation we analysed the presence of caveolin-1 in the DRM fractions as a function of pH. We found that changing the pH did not influence the DRM association, as around 80% of caveolin-1 was DRM associated under all conditions (data not shown). Based on this experiment we conclude that optimal DRM association of C1 is depending on a mildly acidic pH.

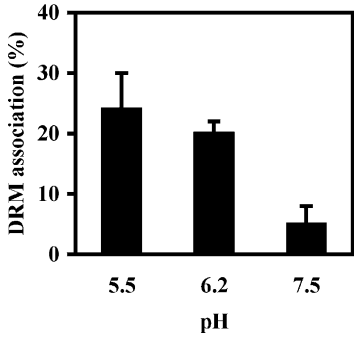

Fig. 4. C1 associates with DRMs in a pH-dependent manner. MDCK cells stably expressing recombinant C1 were extracted with 1% Triton X-100 at 4 °C in 25 mM HEPES; 25 mM MES; and 150 mM NaCl adjusted to different pH (i.e., 5.5, 6.2, and 7.5); and centrifuged overnight to equilibrium in a sucrose step-gradient. Fractions were collected from the top of the gradient. Fraction 1–4 contains the Triton X-100 soluble material, while fraction 5–12 are the lower density fractions containing the Triton X-100 insoluble material. C1 from each fraction were determined by SDS–PAGE, immunoblotting and, subsequently, quantitated using a FujiFilm chemiluminescence camera (see Materials and methods). The bars show Triton X-100 insoluble material × 100/[Triton X-100 insoluble material + Triton X-100 soluble material]. Means ± SD is shown ($n = 3$).

Discussion

In the present work we have studied the possible role of DRMs in the apical targeting of members of the serpin family. We found that the apically secreted serpin C1 to a significant degree could be found associated to DRMs in a pH-dependent manner. Treatment with lovastatin and m-β-CD abolished raft association and influenced secretion of C1. No DRM association could be detected for AT, a serpin secreted in a non-polarised manner from MDCK cells. However, a chimera containing the amino terminal portion of C1 and the carboxyl terminal portion of AT, known to be secreted mainly apically from MDCK cells, was shown not to be DRM associated, demonstrating that the amino terminal of C1 confers apical targeting ability to the chimera but not DRM association. There is thus no correlation between apical targeting and DRM association. Likewise another chimera consisting of the amino terminal portion of α1 and the carboxyl terminus of AT that previously was shown to be secreted mainly apically [19] was shown not to be DRM associated, confirming the lack of correlation between apical targeting and DRM association.

In case apical sorting signals directly or indirectly play an important role in associating apical targeted molecules to DRM, it would be expected that C1 and $C1_{1–132}$AT would associate to DRM in a similar manner. This is however not the case. Thus, association with DRMs and apical targeting of serpins in MDCK cells are not correlated. A similar lack of correlation between apical targeting and DRM association has previously been observed for membrane bound proteins. For lac-

tase and maltase–glycoamylase [38] and mutant forms of hemagglutinin [41] apical targeting has also been shown to be independent of association with DRMs. Furthermore, other mutant forms of hemagglutinin were shown to be associated with DRMs, but not sorted apically [41]. More recently, it was shown that the signals for DRM association and apical targeting of influenza virus neuraminidase are distinct and can vary independently [42]. In addition, two GPI-anchored proteins, both DRM associated, were targeted to both the apical and basolateral membranes in a non-polarised manner [43]. Together, this suggests that neither secretory nor membrane bound proteins necessarily need to use DRMs as apical targeting platforms.

In a functional study, we used lovastatin and m-β-CD to deplete MDCK cells for cholesterol. This treatment prevents formation of DRMs. Cholesterol-depleted MDCK cells fail to target C1 and the endogenously expressed gp80/clusterin to the apical medium (Fig. 2). Based on our results we cannot discriminate whether this is a direct effect on the sorting machinery or an indirect effect on the rate of transport to the apical membrane. In fact, previous observations favour the latter explanation, as treatment with lovastatin/m-β-CD has been shown to affect the rate of apical secretion of thyroglobulin [31], the apical transport of aminopeptidase N in enterocytes [44], and the formation of secretory vesicles from the TGN [45] by blocking the last steps of the budding process and the fission step, as similarly observed for clathrin-coated endocytosis [46]. In addition, it was recently reported that cholesterol depletion of MDCK cells expressing the normally non-polarised protein, rat growth hormone, caused a marked shift in secretion towards a more basolateral distribution [47]. Together, this strongly suggests that cholesterol depletion slows the rate of apical secretion, thereby mediating an apparent non-polarised secretion of apically targeted proteins.

Our result showing that optimal DRM association of C1 is depending on a mildly acidic pH could explain why previous studies [32] did not observe the apically secreted protein gp80/clusterin associated with DRMs, as this study used Triton X-100 extraction buffer with a neutral pH (i.e., 7.5). However, it should be noted that a mildly acidic pH is not a general requirement for DRM association as thyroglobulin is DRM associated at pH 7.5 [31]. The fact that gp80/clusterin is apically secreted, but not associated with DRMs, led to the suggestion that apical targeting of the protein takes place by a DRM-independent mechanism [32], even though its apical targeting requires intact DRMs [33]. Thus, this apparent discrepancy could be a result of not using optimal Triton X-100 buffer conditions.

In conclusion, we report that apically secreted C1 associates with DRMs in a pH-dependent manner, whereas the non-sorted AT does not associate with DRMs. However, association to DRMs is not essential for apical targeting as two apically targeted chimeras were not DRM associated, implying that, for secretory proteins, apical targeting and DRM association are two independent events.

Acknowledgments

Dr. S.C. Bock (Temple University School of Medicine, USA) is thanked for donating the C1 cDNA. Dr. W. Sheffield (McMaster University, Canada) is thanked for donating the AT cDNA and antibodies. We thank Dr. Michael Danielsen and technician Lissi Immerdal (University of Copenhagen, Denmark) for initial help with preparation of glycolipid rafts. We are grateful to Dr. Patrick Keller (EMBL, Germany) for his help with the cholesterol depletion experiments. This project was supported by the Novo Nordisk Foundation and the Danish Medical Research Council. The project was part of a program under the Biomembrane Research Center, Denmark.

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