Secretion of Antithrombin Is Converted from Nonpolarized to Apical by Exchanging Its Amino Terminus for That of Apically Secreted Family Members*

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The three members of the serpin family, corticosteroid binding globulin, α1-antitrypsin, and C1 inhibitor are secreted apically from Madin-Darby canine kidney (MDCK) cells, whereas two homologous family members, antithrombin and plasminogen activator inhibitor-1, are secreted in a nonpolarized fashion. cDNAs coding for chimeras composed of complementary portions of an apically targeted serpin and a nonsorted serpin were generated, expressed in MDCK cells, and the ratio between apical and basolateral secretion was analyzed. These experiments identified an amino-terminal sequence of corticosteroid binding globulin (residues 1–19) that is sufficient to direct a chimera with antithrombin mainly to the apical side. A deletion/mutagenesis analysis showed that no individual amino acid is absolutely required for the apical targeting ability of amino acids 1–30 of corticosteroid binding globulin. The corresponding amino-terminal sequences of α1-antitrypsin and C1 inhibitor were also sufficient to confer apical sorting. Based on our results we suggest that the apical targeting ability is encoded in the conformation of the protein.

In epithelial cells, plasma membrane and secretory proteins are sorted in a polarized manner either to the apical or the basolateral surface. Sorting signals specifying basolateral sorting have been identified in the cytoplasmic domains of many membrane proteins (1, 2). Most of them are characterized either by essential tyrosine (3) or dileucine motifs (4). There is evidence that basolateral sorting of the receptors for transferrin and low density lipoproteins is mediated by AP-1 clathrin adaptors with an epithelia-specific isoform of subunit μ1 (μ1B; Ref. 5). Much less is known about the signals and mechanisms of apical sorting. Apical signals appear to be localized to the noncytosolic segments of membrane proteins, since truncation mutants lacking the cytosolic and transmembrane portions were generally found to retain apical polarity (6–9). Recently, a role of the transmembrane domains in apical sorting has been demonstrated for several proteins (10–13). Also motifs in the cytoplasmic tails of rhodopsin (14) and the Na⁺-dependent bile acid transporter (15, 16) have been shown to mediate apical sorting. With respect to polarized secretion of secretory proteins, it has been shown, for example, that Madin-Darby canine kidney (MDCK) cells secrete gp80/clusterin (17), erythropoietin (18), and corticosteroid binding globulin (CBG) (19) mainly from the apical side, whereas a range of proteins, including growth hormone, lysozyme, prochymosin, the immunoglobulin κ chain (20), cystatin C (6), and urotoglobin (21), are secreted equally from both plasma membrane domains. It was shown that N-linked glycans can act as apical targeting signals for secretory proteins, based on the observation that growth hormone, normally secreted in a nonpolarized manner from MDCK cells, is secreted mainly from the apical side after insertion of one or two N-linked glycosylation sites (22). In addition, erythropoietin, which is normally secreted at the apical side of MDCK cells, is secreted in a nonpolarized manner after mutation of two distinct N-linked glycosylation consensus sequences (18). However, N-linked glycosylation is not the only apical targeting signal for secretory proteins, since mutagenesis of the six N-glycosylation sites of CBG, individually or together, did not affect its apical secretion from MDCK cells (23). Other examples where mutation of the only N-glycosylation site did not affect the polarity of secretion are the Hepatitis B surface antigen (24) and a soluble form of the p75 neurotrophin receptor (25). In addition, chromogranin A is not N-glycosylated but nevertheless is secreted mainly from the apical side of MDCK cells (26). Thus, there seem to be at least two mechanisms of apical sorting of secretory proteins, either dependent or independent of N-glycans.

To characterize N-glycan-independent apical sorting, we studied members of the serpin family as model proteins. Some of them, including CBG (19) 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), are secreted in a non-sorted manner (27). The structural similarity allowed the construction of chimeras to identify the sequence requirements for apical sorting. In the present study we have shown that amino-terminal segments of the three serpins, CBG, α1, and C1, are sufficient to direct AT to the apical side of MDCK cells. A combined deletion and mutagenesis analysis showed that no

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1 The abbreviations used are: MDCK, Madin-Darby canine kidney; AT, antithrombin; α1, α1-antitrypsin; CBG, corticosteroid binding globulin; C1, C1 inhibitor; PAI-1, plasminogen activator inhibitor-1.
individual amino acid is essential for the ability of residues 1–30 of CBG to direct AT mainly apically from MDCK cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells (strain II), a kind gift from K. Mostov (University of California), were maintained and transfected as described previously (6). COS-1 cells were grown in modified minimal essential medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-1 cells were transfected using FuGENETM (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. For sorting experiments, 10^6 cells per well were seeded onto Transwell filters (Costar Europe Ltd., Badhoevedorp, The Nederlands; 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 judged by assessing the tightness of the monolayer (28). Under these conditions the monolayers have a transepithelial resistance of ~450 ohms cm^2 measured by a MilliliMF-2 voltohmeter (Millipore Continental Water Systems, Bedford, MA).

DNA Constructs—The pcDM/CMV vector was used for recombinant expression of all constructs. cDNAs encoding human CBG (29), rabbit AT (30), human α1 (31), and human C1 (32) were used. Chimeras were generated using standard DNA techniques. The rat enkephalin signal sequence (33) was used in the following constructs: ΔAT_{36–419}, ΔAT_{413–419}, ΔCBG_{6–30}AT, and ΔCBG_{12–30}AT. ΔCBG_{6–30}AT and ΔAT_{413–419} were constructed so they contain the same 3′- and 5′-untranslated regions, the same cleavable signal peptide, and apart from the amino-terminal 25 amino acids, the same coding sequence. All cDNA constructs were verified by sequencing. Numbers in construct names refer to amino acid number of the mature protein after signal peptide cleavage.

Metabolic Labeling of Cells and Immunoprecipitation—Confluent MDCK cells seeded on filters were preincubated for 30 min in methionine-free medium and thereafter pulse-labeled with 500 μCi/ml of [35S]methionine for 30 min. The cells were grown to confluence on filters, pulse-labeled with [35S]methionine, and chased for 4 h. Apical (Ap) and basolateral (Ba) media were collected separately and individually immunoprecipitated in various dilutions (×2 and ×4) with a fixed amount of antibodies. The immunoprecipitates were analyzed on NuPAGE gels (right panel). Only when the dilution resulted in a corresponding reduction in the signal (as in the experiments shown) was it assumed that the immunoprecipitation was quantitative, and the result was quantitated using a phosphorimager (left panel). Bars represent mean ± S.D. for 5–10 experiments.

RESULTS

Secretion Is Apical for CBG, but Nonpolarized for AT—Three members of the serpin family (CBG, α1, C1) are secreted apically from MDCK cells, whereas AT and PAI1 are secreted in a nonpolarized manner (19, 27). To identify the apical targeting determinant we generated chimeras between an apically targeted member of the family and a nonsorted member. We first chose CBG and AT, which have 33% identity and 45% similarity, and for which good antibodies were available.

It has previously been observed that stable transfected cell lines expressing CBG showed some variation in the efficiency of apical secretion in the range of 69–83% apical (19). To eliminate clonal variation, transfected G418-resistant cells expressing CBG (approximately 200 independent clones) were pooled. The cells were grown to confluence on filters, pulse-labeled for 20 min with [35S]methionine, and chased for 4 h. The apical and basolateral media were collected, subjected to immunoprecipitation, and analyzed by NuPAGE-gel electrophoresis. As shown in Fig. 1, wild-type CBG was secreted in a polarized manner from a pool of MDCK clones with an apical/basolateral ratio of 78/22. Wild-type CBG (and some of the chimeras, see below) runs as multiple bands due to partial utilization of three of its six N-glycosylation sites (36). Essentially the same results were obtained in three independent transfections. This is very similar to what had been obtained for individually isolated clones of MDCK cells expressing the protein (19, 23). Recombinantly expressed AT was secreted from a pool of clones at an apical/basolateral ratio of 44/56 (Fig. 1), which is similar to the result obtained when individual isolated clones were analyzed (27). On this basis we decided to analyze pools of clones instead of individual clones throughout this study.

The Amino-terminal Sequence of CBG Is Sufficient for Apical Targeting—Initially a series of eight chimeras was generated between CBG and AT. The first chimera consisted of the amino

FIG. 1. Apical targeting correlates with the presence of the amino-terminal end of CBG. Pools of MDCK cells expressing CBG, AT, CBG_{30AT}, CBG_{30AT}, or CBG_{19AT} or CBG_{19AT} were seeded on filters. Tight filter-grown cells were used for experiments shown. It has previously been observed that stable transfected clonal cell lines expressing CBG showed some variation in the efficiency of apical secretion in the range of 69–83% apical (19). To eliminate clonal variation, transfected G418-resistant cells expressing CBG (approximately 200 independent clones) were pooled. The cells were grown to confluence on filters, pulse-labeled for 20 min with [35S]methionine, and chased for 4 h. The apical and basolateral media were collected, subjected to immunoprecipitation, and analyzed by NuPAGE-gel electrophoresis. As shown in Fig. 1, wild-type CBG was secreted in a polarized manner from a pool of MDCK clones with an apical/basolateral ratio of 78/22. Wild-type CBG (and some of the chimeras, see below) runs as multiple bands due to partial utilization of three of its six N-glycosylation sites (36). Essentially the same results were obtained in three independent transfections. This is very similar to what had been obtained for individually isolated clones of MDCK cells expressing the protein (19, 23). Recombinantly expressed AT was secreted from a pool of clones at an apical/basolateral ratio of 44/56 (Fig. 1), which is similar to the result obtained when individual isolated clones were analyzed (27). On this basis we decided to analyze pools of clones instead of individual clones throughout this study.

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terminus of CBG, including the signal peptide and amino acids 1–43 of the mature protein fused in-frame with the complementary carboxyl terminus of AT (amino acids 80–419) (Fig. 2). This construct was named CBG_{1–43}AT. The reverse construct containing the signal peptide and the amino-terminal part of AT up to amino acid 79 (Fig. 2) followed by the complementary carboxyl terminus portion of CBG was correspondingly named AT-CBG_{44–383}. CBG_{1–43}AT and AT-CBG_{44–383} thus are each others inverted chimera. We also constructed CBG_{113}AT, AT-CBG_{114–383}, CBG_{1–216}AT, AT-CBG_{114–383}, CBG_{1–297}AT, and AT-CBG_{298–383}, a total of four pairs of inverted chimeras.

As a first test of expression and secretion, the eight chimeras and wild-type CBG and AT were transiently transfected into COS-1 cells. All constructs were expressed. However, after a 20-min pulse and a 4-h chase, only wild-type AT, wild-type CBG, and the chimera CBG_{1–43}AT were efficiently secreted (data not shown), indicating that the other seven chimeras were not efficiently folded in the endoplasmic reticulum. We did not study these seven chimeras further. All constructs described in this study were tested for expression and secretion efficiency in COS-1 cells and/or MDCK cells in this manner. Only constructs with expression and secretion efficiency between the two wild-type proteins were analyzed further unless otherwise stated.

When expressed in MDCK cells and analyzed on filters as described above, CBG_{1–43}AT was secreted predominantly from the apical surface, with an apical/basolateral ratio of 79/21 (Fig. 1). The amino-terminal 43 amino acids of CBG are thus sufficient to confer apical targeting to the chimeric protein. To further define the peptide sufficient for apical targeting, we constructed three new chimeras, CBG_{1–34}AT, CBG_{1–30}AT, and CBG_{1–19}AT (Fig. 2). In MDCK cells, all three chimeric proteins were secreted mainly from the apical side (Fig. 1) with apical/basolateral ratios of 81/19, 85/15, and 78/25 for CBG_{1–34}AT, CBG_{1–30}AT, and CBG_{1–19}AT, respectively. These experiments narrowed the sequence sufficient to confer apical targeting down to residues 1–19 of CBG.

The Apical Targeting of Chimeras Is Not Due to Truncation of the Amino Termminus of AT—The chimeric constructs described so far are all shorter at the amino terminus than AT, which has an amino-terminal extension compared with CBG. To investigate whether the apical secretion of the chimeric molecules is due to the peptide sequence transferred from CBG or to the truncation of the molecule, the 35 amino-terminal residues of AT were deleted by fusing a cleavable signal peptide in front of amino acids 36–419 of AT. The resulting construct was named AT_{36–419} (Fig. 2). In a second version of this construct, AT_{41–419}, an additional 5 amino acids were deleted at the amino terminus (Fig. 2). Upon expression in filter-grown MDCK cells AT_{36–419} was secreted with an apical/basolateral ratio of 50/50 and AT_{41–419} with an apical/basolateral ratio of 52/48 (data not shown). As a control, the secretion of gp80/clusterin, an endogenous apical secretory protein, was also examined and found to be secreted mainly apically from the cells expressing AT_{36–419} and AT_{41–419} (data not shown). These cells thus have not lost their ability to target proteins to the apical side. Truncation of the amino terminus of AT alone is therefore not enough to induce apical targeting of the molecule.

The Amino-terminal Sequences of α1 and C1 Are Also Sufficient for Apical Targeting of Chimeras with AT—Like CBG, the serpins α1 and C1 are also secreted mainly apically from MDCK cells (27). An alignment of the amino-terminal sequences of these three serpins and the two nonsorted serpins, AT and PAI-1 (Fig. 3A), shows a big diversity in length and sequence, also when only the apically secreted serpins are compared. To determine whether the amino terminus of α1 or C1 can, like that of CBG, direct a chimera with AT into the apical pathway, we constructed the two chimeras, α1_{1–39}AT and C1_{1–132}AT, consisting of amino acids 1–39 of α1 and 1–132 of C1, respectively, fused to their corresponding carboxyl terminus of AT. α1_{1–39}AT was fused at the same point as CBG_{1–30}AT, whereas the fusion point of C1_{1–132}AT was one amino acid further toward the amino terminus. Both constructs were secreted mainly to the apical side with an apical/basolateral ratio of 71/29 for α1_{1–39}AT and of 75/25 for C1_{1–132}AT (Fig. 3B). The amino-terminal portions of α1 and C1 are thus equally capable of conferring apical targeting to AT as that of CBG.

Apical Targeting by Residues 1–30 of CBG Does Not Depend on Any Individual Amino Acid—We investigated the significance of residues 1–30 of CBG for apical targeting more in detail. To test whether amino acids 1–5 of CBG are important, we made a construct containing a cleavable signal peptide and amino acids 6–30 of CBG followed by the complementary car-
Apical Targeting of Serpins

**Fig. 3. The amino termini of α1 and C1 are able to confer apical targeting to AT.** A, alignment of the amino-terminal sequences of the apical serpins C1, α1, and CBG and of the nonpolarized serpins AT and PAI-1. The 19 residues of CBG sufficient for apical sorting are underlined. The fusion points for α1–20AT and C11–23AT are indicated by arrows. B, filter-grown cells expressing α1–23AT or C11–23AT were analyzed as described in the legend to Fig. 1. Bars represent mean ± S.D. for nine experiments.

To investigate the importance of each individual amino acid in the sequence 6–30 of CBG for the apical targeting ability, we mutated individual amino acids in the CBG1–30AT construct to the amino acid present at the corresponding position in AT according to our alignment shown in Fig. 3. When comparing the sequence 6–30 of CBG and the corresponding sequence of AT, there are 7 positions where the two proteins have identical amino acids. Thus a total of 18 mutants were constructed. Upon expression in MDCK cells all but one of the mutant proteins (CBG1–30AT H14V) were efficiently secreted into the media and all of them with essentially the same polarity as CBG1–30AT (Fig. 5). The mutation H14V was instead constructed in the context of CBG1–19AT, and the resulting protein (CBG1–19AT H14V) was efficiently expressed and secreted predominantly into the apical medium essentially like CBG1–19AT. These results show that no single amino acid is absolutely required for apical targeting of the amino acids 1–30 of CBG.

Residues 20–383 of CBG Are Also Sufficient for Apical Targeting—Our results show that the amino-terminal 19 residues of CBG are sufficient for apical targeting of a chimera with AT. To test whether the amino-terminal sequence is the only determinant of polarity, AT-CBG20–383 i.e. the inverted chimera of CBG1–19AT, was constructed. AT-CBG20–383 was secreted apically from MDCK cells at an apical/basolateral ratio of 70/30 (Fig. 4). This result shows that the carboxyl terminus of CBG contains additional determinants conferring apical targeting to a chimeric protein. Similarly, AT-CBG31–383, the inverted chimera of CBG1–30AT, was secreted mainly to the apical side of MDCK cells, but at a lower rate than wild-type CBG (data not shown).

**DISCUSSION**

Polarized secretion might be generated by signal-mediated delivery to one surface and a default pathway to the other. Alternatively, delivery to both destinations may be signal-mediated. It was recently shown that the apical sorting machinery in RPE-J cells could be saturated by overexpression (37), strongly suggesting that apical secretion is signal-mediated at least in this cell line.

In the present study, we analyzed the sorting of members of the serpin family that are either targeted predominantly to the apical side of MDCK monolayers (CBG, α1, and C1) or secreted in a nonpolarized manner (AT and PAI-1). Analyzing chimeras between CBG and AT, we found that a short peptide derived from the amino-terminal end of CBG is able to confer apical targeting to the recombinant proteins. Apical secretion was not due to truncation of the 35-amino acid amino-terminal extension present in AT and lacking in CBG, since its deletion did not result in apical targeting. The amino-terminal extension of AT thus does not carry basolateral sorting information or hide apical sorting information.

Amino acids 1–19 or 6–30 of CBG are able to confer apical targeting, suggesting that the determinant is contained within residues 6–19 of CBG. The amino-terminal sequences of α1 (residues 1–39) and of C1 (residues 1–132) are also able to confer apical targeting to the corresponding AT fusion proteins. Comparison of these sequences reveals hardly any conservation on the amino acid level. There is only one conserved position, a hydrophobic residue at the position corresponding to residue 17 of CBG. However, the two nonsorted serpins also have a hydrophobic amino acid at this position (Leu in AT and Val in PAI-1). The presence of a nonpolar side chain at this position is thus likely to be preferred in the serpin fold, but unlikely to be crucial for apical targeting. We are thus unable to identify a peptide motif putatively responsible for apical targeting. A combined deletion and mutagenesis analysis showed that no single amino acid in residues 1–30 of CBG is absolutely required for apical targeting. However, as both residues 1–19 and 20–383 of CBG are able to confer apical tar-
targeting ability, it is formally possible, albeit not very likely, that residues 1–30 of CBG contain two apical determinants, and consequently the mutagenesis analysis would miss identifying them. However, the lack of conservation between the aminoterminals of CBG, α1, and C1 suggest that apical targeting is not mediated by a conserved linear amino acid sequence.
This situation is reminiscent of that recently described for the apical sorting of the α-subunit of the gastric H,K-ATPase. Analysis of chimeras between the α-subunits of the apical H,K-ATPase and of the homologous basolateral Na,K-ATPase identified the predicted fourth transmembrane domain (TM4) of the H,K-ATPase as sufficient for apical sorting in LLC-PK1 cells (13). Furthermore, the two flanking regions of TM4 were also able to redirect the protein to the apical side of the cells, whereas either one of the flanking sequences alone was not (13). The flanking sequences were proposed to induce a conformational change in TM4 of the Na,K-ATPase, mimicking the conformation of TM4 of the H,K-ATPase. Alternatively, TM4 or the flanking sequences of the H,K-ATPase may impose certain conformations on other parts of the protein, which could then be recognized by components of the apical sorting machinery. In a similar manner the serpins analyzed here may carry a conformation-dependent apical sorting signal either in the amino terminus or elsewhere in the protein.

In the present study we have shown that not only amino acids 1–19 of CBG carry apical targeting information, but also amino acids 20–383. Redundant signals thus appear to ensure correct cellular localization of CBG, comparable with the low density lipoprotein receptor and CD1d, which was shown to have multiple basolateral sorting signals (38, 39). Also influenza virus neuraminidase, a transmembrane protein, possesses two apical determinants: one in the ectodomain (10) and the other in the transmembrane domain (40).

Residues 1–30 of CBG contain one consensus sequence for N-glycosylation at Asn, but mutation of this site in the context of wild-type CBG (23) or in the context of CBG1–30AT (CBG1–30AT N9A or CBG1–30AT S11N) did not disturb apical targeting of the recombinant product. As CBG may have redundant apical targeting information, even within residues 1–30, N-glycosylation alone is not the apical determinant, but it cannot be excluded that N-glycosylation serves as an apical targeting determinant in addition to a determinant of a different nature. CBG does not carry O-linked glycosylation, which had been suggested to act as an apical determinant for the neurotrophin receptor (25).

It cannot be formally excluded that the effect of the amino-terminal sequence of CBG on sorting is not due to the introduction of an apical sorting determinant, but is instead attributable to the disruption of a weak basolateral localization signal in AT. However, we consider this unlikely, since a number of exogenous proteins from diverse sources lacking polarized sorting are secreted in a nonpolarized manner from MDCK cells (6, 20, 21).

We envision that the apical targeting of serpins is a receptor-mediated process and that the receptors in turn have a regulated intracellular traffic. It has been suggested that detergent-resistant membranes function as apical targeting platforms (41). In addition it was recently shown that the apically targeted, secretory protein thyroglobulin associates with detergent-resistant membranes during transport to the cell membrane (42). However, a correlation between apical targeting and association to the detergent-resistant membrane could not be observed when wild-type serpins and chimeras of serpins were investigated.2 It is thus unlikely that detergent-resistant membranes are involved in the apical transport of serpins.

In this paper we have shown that the exchange of a short NH2-terminal peptide of the nonsorted serpin AT by the nonconserved NH2-terminal peptides derived from three apical members of the serpin family is able to confer apical targeting. On this basis we suggest that polarized sorting of the apically secreted serpins does not depend on a strictly conserved linear sequence but may depend on the protein’s conformation.

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