Apical and non-polarized secretion of serpins from MDCK cells

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Abstract Corticosteroid binding globulin, a member of the serpin family, was previously shown to be secreted mainly apically from MDCK cells in an *N*-glycan independent manner [Larsen et al. (1999) FEBS Lett. 451, 19–22]. Apart from *N*-glycosylation, serpins are not known to carry any other posttranslational modifications, suggesting the presence of a proteinaceous apical sorting signal. In the present study we have expressed four other members of the serpin family: α1-antitrypsin, C1 inhibitor, plasminogen activator inhibitor-1 and antithrombin in MDCK cells. Tight monolayers of transfected cells were grown on filters and the amounts of recombinantly expressed serpins in the apical and the basolateral media were determined. α1-Antitrypsin and C1 inhibitor were found mainly in the apical medium whereas plasminogen activator inhibitor-1 and antithrombin were found in roughly equal amounts in the apical and basolateral media. Control experiments showed that all four serpins are transported along the exocytotic pathway in an uncomplicated way that does not involve transcytosis or differences in stability on the two sides of the cells. We conclude that some members of the serpin family including corticosteroid binding globulin, α1-antitrypsin and C1 inhibitor are secreted mainly apically from MDCK cells whereas plasminogen activator inhibitor-1 and antithrombin are secreted in a non-polarized manner.

Key words: Serpin; MDCK cell; Apical secretion; Non-sorted secretion

1. Introduction

Polarized epithelial cells are able to secrete proteins specifically at the apical or basolateral plasma membrane and to express membrane-bound proteins specifically at the apical or basolateral plasma membrane. It was recently suggested that *N*-glycans act as an apical sorting signal for secretory proteins [1]. This was based on the observation that rat growth hormone, which is normally secreted randomly from MDCK cells, is secreted from the apical side after introduction of one or two *N*-glycosylation sites [1]. Surface expression of a GPI-anchored version showed that *N*-glycosylation may also be important for the apical delivery of GPI-anchored proteins to the apical plasma membrane [2]. In addition *N*-glycans have also been shown to be important for the apical targeting of erythropoietin [3] in MDCK cells. Whether *N*-glycans directly function as apical targeting signals or whether they play a facilitative role, which allows e.g. a proteinaceous apical sorting signal to be presented correctly, is at present unclear [4].

Several examples have, however, been described where *N*-glycans cannot be responsible for apical targeting. These include the apical secretion of corticosteroid binding globulin (CBG) from MDCK cells, since six mutants, each lacking one of the *N*-glycosylation sites, and a mutant lacking all six *N*-glycosylation sites are secreted to the apical side of MDCK cells [5]. Other examples are the hepatitis B surface antigen [6] and a soluble form of p75 neurotrophin receptor [7] where mutation of the only *N*-glycosylation site did not affect the polarity of secretion. Also, tunicamycin has been used to study the role of *N*-glycans in apical targeting. These studies are, however, difficult to interpret since tunicamycin abolished the apical secretion of CBG mutated in all six *N*-glycosylation sites [5], suggesting that an essential component of the apical targeting machinery is tunicamycin sensitive.

CBG belongs to the large serpin (serine proteinase inhibitors) family [8]. The serpins are globular proteins about 400 residues long. They are similar in their three-dimensional structure and contain three β-sheets (sA, sB, and sC) and nine α-helices (hA–hI). The three-dimensional structure of several serpins has been determined, for example α1-antitrypsin (α1-AT) [9,10], plasminogen activator inhibitor-1 (PAI-1) [11,12], and antithrombin (AT) [13]. From these studies it is clear that serpins can undergo a profound conformational change where the region around the scissile bond forms an additional strand within the β-sheet denoted sA [14]. The members of the serpin family share around 30% sequence identity with each other.

Another member of the serpin family, α1-AT, is, after recombinant expression, secreted mainly to the apical side of the human bronchial epithelial cell line, Bet-1A [15]. In the present study we have attempted to extract information about the apical sorting signal on CBG by exploiting the diversity of the serpin family. Four members of the serpin family were recombinantly expressed in MDCK cells and the polarity of secretion was examined. Much to our surprise we found that two serpins, PAI-1 and AT, are secreted in a non-polarized manner, whereas α1-AT and C1 inhibitor (C1-I) are secreted mainly apically from MDCK cells.

2. Materials and methods

2.1. Cell culture

MDCK cells (strain II), a kind gift from K. Mostov (University of California, USA), were grown and transfected as described [16]. An expression vector containing human AT cDNA (pMLP-α1AT), described in [17], was a generous gift from R.G. Crystal (Cornell Medical Center, USA). The human C1-I cDNA [18] was a generous gift from S.C. Bock (Temple University School of Medicine, USA). The C1-I cDNA was subcloned into the pTEJ-4 vector [19]. The pTEJ-5

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vector containing the human PAI-1 cDNA [19] was a generous gift from Dr. T.W. Schwartz (University of Copenhagen, Denmark). No induction of the promoter with e.g. $CdCl_2$ was used. The rabbit AT cDNA [20] was a kind gift from W.P. Sheffield (McMaster University, Canada) and was subcloned into the pTEJ-4 vector [19]. Stable clones were selected using 0.65 mg/ml G418 and isolated. Cells were grown on Transwell chambers No. 3412 (Costar Europe Ltd., Badhoevedorp, the Netherlands) allowing separate access to the apical and basolateral membrane. Filter grown cells were used for experiments after confluence as indicated by their tightness, assayed as described [21]. Under these conditions the cell monolayer has a transepithelial resistance of $\sim$450 Ω/cm^2 (measured by a Millicell-ERS voltohmmeter, Millipore Continental Water Systems, Bedford, MA, USA).

2.2. Metabolic labelling

Cells were labelled with [^{35}S]methionine as described [22] and immunoprecipitated essentially as described [22]. α1-AT was detected using a rabbit anti-human α1-AT antibody (Boehringer Mannheim No. 605 002). C1-I was detected using a rabbit anti-human C1-I antibody A0253 (Dako, Copenhagen, Denmark). PAI-1 was detected using a PAI-1 clone 1 antibody (kindly provided by P.A. Andreasen, University of Århus, Denmark). AT was detected using affinity purified sheep anti-rabbit AT IgG (a generous gift from W.P. Sheffield, McMaster University, Canada). Polyclonal antibodies against human gp80 (clusterin) were a generous gift from C. Koch-Brandt, J. Gutenberg Universität, Germany. Immunoprecipitates were washed in phosphate buffered saline containing 1% Triton X-100, 5 mM EDTA and 0.5% DOC. The immunoprecipitates were analyzed on 10% SDS–PAGE gels modified accordingly to [23] or NuPAGE gels (Novex, San Diego, CA, USA)

2.3. Quantitative immunoprecipitation

To quantitate the secretion of the recombinant proteins on the apical and basolateral side quantitative immunoprecipitation was used as previously described [5]. Each sample was diluted by a series of two-fold dilutions and each dilution was immunoprecipitated separately and analyzed on NuPAGE gels. It was assumed that the immunoprecipitation was quantitative and the result was quantitated using a phosphoimager, only if the two-fold dilution also resulted in a two-fold reduction of the signal.

2.4. Other methods

PAI-1 was quantitated using ELISA performed as described for PAI-1 ELISA II [24]. Tunicamycin was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Control cells were treated with similar concentrations of DMSO.

3. Results

3.1. Recombinantly expressed α1-AT and C1-I are secreted mainly to the apical side of MDCK cells

MDCK cells were transfected with an expression vector containing the human α1-AT or the human C1-I cDNA and G-418 resistant clones were isolated. The clones were screened for α1-AT or C1-I expression using metabolic labelling with [^{35}S]methionine followed by immunoprecipitation and analysis on NuPAGE gels. Expression of α1-AT could be detected in media from transfected MDCK cells (Fig. 1, lane 1), but not in media from non-transfected MDCK cells (Fig. 1, lane 2). The immunoprecipitated α1-AT showed an apparent molecular weight around 50 kDa as expected. Expression of C1-I could be detected in media from transfected MDCK cells (Fig. 1, lane 3), but not in media from non-transfected MDCK cells (Fig. 1, lane 4). The circulating C1-I has an apparent molecular weight of 104 000 Da [18] as was also observed for the recombinantly expressed protein (Fig. 1, lane 3). Occasionally immunoprecipitated C1-I appeared as a doublet band (as in Fig. 2) and occasionally as a single band (as in Fig. 1). This is probably due to differences in the glycosylation pattern.

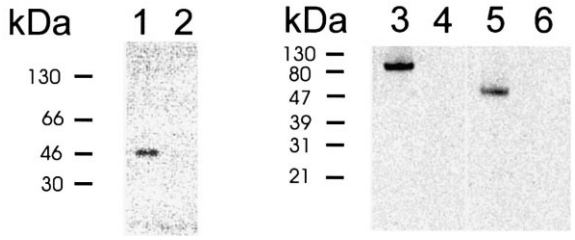

Fig. 1. Recombinant expression of serpins in MDCK cells. Transfected and non-transfected cells were labelled with [^{35}S]methionine for 20 min and chased for 4 h. The media were collected and immunoprecipitated and analyzed on 10% NuPAGE gels. Media from cells expressing α1-AT (lane 1) and non-transfected cells (lane 2) were immunoprecipitated with antibodies against α1-AT. Media from cells expressing C1-I (lane 3) and non-transfected cells (lane 4) were precipitated with antibodies against C1-I and media from cells expressing AT (lane 5) and non-transfected cells (lane 6) were precipitated with antibodies against AT. The positions of marker proteins and their molecular masses in kDa are indicated.

MDCK cells expressing α1-AT or C1-I 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, subjected to quantitative immunoprecipitation and analyzed on NuPAGE gels as shown (Fig. 2, upper and middle panels). The apical/basolateral ratio was determined using a phosphoimager and showed (Fig. 3A) that α1-AT is secreted in a polarized way from MDCK cells at an apical/basolateral ratio of 81/19 (S.D. ±5, $n=11$) and that C1-I is secreted mainly to the apical side from MDCK cells (Fig. 3B) at an apical/basolateral ratio of 70/30 (S.D. ±4, $n=8$). A high and a low expression clone were investigated with essentially the same result.

3.2. Recombinantly expressed PAI-1 is secreted in a non-polarized manner from MDCK cells

MDCK cells transfected with an expression vector containing the complete PAI-1 cDNA were also investigated. G-418 resistant clones were isolated and their expression of PAI-1

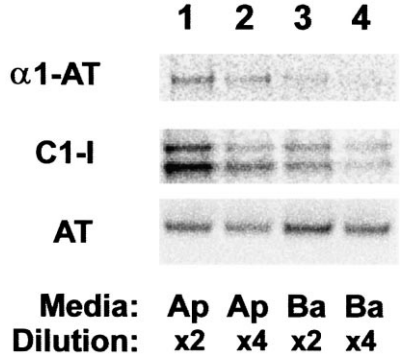

Fig. 2. Immunoprecipitation of α1-AT, C1-I and AT is quantitative. Transfected MDCK cells expressing either α1-AT, C1-I or AT were grown to confluence on filters. The cells were then labelled for 20 min with [^{35}S]methionine and chased for 4 h and apical (Ap) or basolateral (Ba) media were collected and individually immunoprecipitated in various dilutions (in this case ×2 and ×4) with a fixed amount of antibodies. The immunoprecipitates were analyzed on NuPAGE gels. Only when the dilution resulted in a corresponding reduction in the signal (as in the experiment shown) was it assumed that the immunoprecipitation was quantitative and the result was quantitated using a phosphoimager.

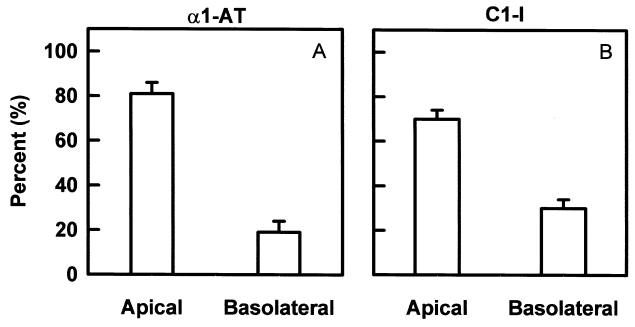

Fig. 3. α1-AT and C1-I are secreted mainly to the apical side of MDCK cells. MDCK cells expressing either α1-AT (A) or C1-I (B) were grown to confluence on filters, labelled with [^{35}S]methionine and chased for 4 h. The media were collected individually from the apical and basolateral side and quantitated via immunoprecipitation (see Fig. 2). The amount of apical or basolateral secretion is shown as a percentage of the total secreted α1-AT or C1-I. Mean ± S.D. is shown.

was examined by measuring the PAI-1 concentration in the media after 24 h incubation using ELISA. Little or no PAI-1 could be detected in media from non-transfected cells, whereas several of the clones of transfected cells showed clearly detectable levels of PAI-1 (~15 ng/ml).

Two clones expressing PAI-1 were grown until confluence on filters and media were collected from both the apical and the basolateral side after 6 h. The amount of PAI-1 in the media was then determined by ELISA. The PAI-1 was distributed as expected for a non-sorted protein at an apical/basolateral ratio of 31/69 (S.D. ±4, $n=9$) (Fig. 4A). The results obtained from the two clones were essentially the same. Similar results were obtained when the media were collected from the cells after 4 or 24 h. In order to verify that the clones investigated had not lost their ability to secrete a protein to the apical side, the secretion of the endogenous protein gp80 was investigated. Gp80 has previously been shown to be secreted mainly at the apical side of MDCK cells [25]. Tight filter grown transfected cells were pulsed for 20 min with [^{35}S]methionine and chased with excess unlabelled methionine for 4 h. The apical and the basolateral media were collected,

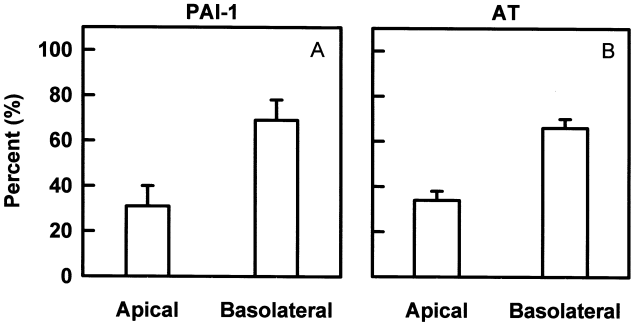

Fig. 4. PAI-1 and AT are secreted from MDCK cells in a non-sorted manner. MDCK cells expressing PAI-1 (A) were grown to confluence on filters. The apical and basolateral media were collected after 6 h and the PAI-1 concentration was determined by ELISA. MDCK cells expressing AT (B) were grown to confluence on filters, labelled with [^{35}S]methionine and chased for 4 h. The media were collected individually from the apical and basolateral side and quantitated via immunoprecipitation (see Fig. 2). The amount of apical or basolateral secretion is shown as a percentage of the total secreted AT. Mean ± S.D. is shown.

immunoprecipitated using antibodies against gp80 and the immunoprecipitate was analyzed on 10% SDS–PAGE gels. The transfected cells secreted gp80 mainly to the apical side (results not shown).

To determine whether PAI-1 is equally stable on the apical and basolateral sides of the cells, 24 h medium from transfected cells was collected. Tight filter grown non-transfected MDCK cells were exposed to the PAI-1 containing medium from both the apical and basolateral side for various periods, after which the media were collected and the concentration of PAI-1 was determined by ELISA. PAI-1 exposed to the apical and the basolateral plasma membrane was equally stable, with a very long half-life (data not shown).

3.3. *Recombinantly expressed AT is secreted in a non-polarized manner from MDCK cells*

Similarly, MDCK cells were transfected with an expression vector containing the rabbit AT cDNA and G-418 resistant clones were isolated. Clones were screened for AT expression as described above for α1-AT and expression could be detected in media from transfected MDCK cells (Fig. 1, lane 5), but not in media from non-transfected MDCK cells (Fig. 1, lane 6).

MDCK cells expressing AT were grown to confluence on Transwell filters and the polarity of the secretion was analyzed as described for α1-AT (Fig. 2, lower panel). Quantitation showed that AT is secreted in a non-polarized manner from MDCK cells (Fig. 4B) at an apical/basolateral ratio of 34/66 (S.D. ±4, $n=11$). A high and a low expression clone were investigated with essentially the same result. As a control the secretion of gp80 was also examined and we found that gp80 is secreted mainly apically from the cells expressing AT (data not shown).

3.4. *No transcytosis of α1-AT, C1-I, PAI-1 or AT occurs in MDCK*

The possibility that MDCK cells take up any of the recombinantly expressed serpins followed by transcytosis either in an apical to basolateral direction or vice versa was investigated. MDCK cells expressing one of the serpins α1-AT, C1-I or AT were labelled with [^{35}S]methionine for 30 min and chased for 4 h. The medium, containing metabolically labelled serpin, was collected, and applied to one side of filter grown tight non-transfected MDCK cells and after 4 h incubation the media from both sides were collected and immunoprecipitated. Less than 5% of the labelled serpin applied to the apical side was found on the basolateral side and less than 5% applied to the basolateral side was found on the apical side.

To examine whether PAI-1 is redistributed by transcytosis after secretion from the cells, 24 h medium was collected from a confluent bottle of cells expressing PAI-1. Such medium contains an equivalent concentration of PAI-1 as is accumulated in 24 h media of tight filter grown cells expressing PAI-1. Tight filter grown non-transfected MDCK cells were exposed to the PAI-1 containing medium from either the apical or the basolateral side for 24 h, after which the media were collected and the concentration of PAI-1 was determined by ELISA. When the PAI-1 containing medium was applied from the apical side approximately 1% of the PAI-1 was found on the basolateral side after 24 h. When applied from the basolateral side approximately 2% was found on the apical side

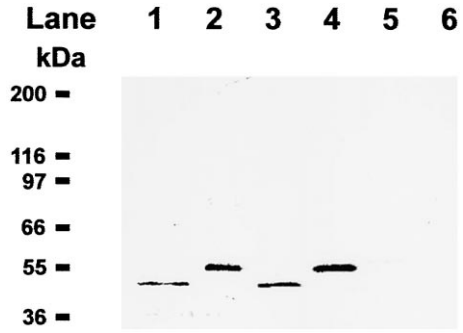

Fig. 5. PAI-1 is *N*-linked glycosylated. Pulse labelled PAI-1 was immunoprecipitated and incubated in the presence (lane 1) or absence (lane 2) of *N*-glycosidase F and analyzed by SDS–PAGE. In an additional experiment transfected (lanes 3 and 4) and non-transfected (lanes 5 and 6) MDCK cells were preincubated in the presence (lanes 3 and 5) or absence (lanes 4 and 6) of tunicamycin for 1 h, labelled with [^{35}S]methionine for 20 min and chased for 4 h still in the presence or absence of tunicamycin. The media were collected, immunoprecipitated and analyzed by SDS–PAGE. The positions of marker proteins and their molecular masses in kDa are indicated.

after 24 h. In conclusion, none of the four serpins investigated here is transcytosed from the apical to the basolateral side or vice versa to any significant degree.

3.5. *PAI-1 is secreted in a non-polarized manner but is N-glycosylated*

It is still controversial whether *N*-glycans alone can act as apical sorting signals. We therefore investigated whether the non-sorted serpin PAI-I is *N*-glycosylated when expressed in MDCK cells. Pulse labelled PAI-1 was immunoprecipitated and incubated in the presence or absence of *N*-glycosidase F and analyzed on SDS–PAGE gels. A band of approximately 46 kDa could be detected on the sample of *N*-glycosidase F treated PAI-1 (Fig. 5, lane 1). This is close to the non-glycosylated molecular mass predicted from the amino acid sequence [26]. PAI-1 not treated with *N*-glycosidase F appeared as a band of approximately 55 kDa (Fig. 5, lane 2). This corresponds well with previous findings [27]. Incubation of PAI-1 with *N*-glycosidase F thus caused a clear shift in the molecular weight.

Transfected and non-transfected cells were also preincubated for 1 h in the presence of 15 μg/ml tunicamycin, pulsed for 20 min with [^{35}S]methionine and chased with excess unlabelled methionine for 4 h still in the presence of tunicamycin. The media were collected, immunoprecipitated and the size of the immunoprecipitate was analyzed on SDS–PAGE gels. A similar experiment was carried out in parallel in the absence of tunicamycin. A band of approximately 44 kDa could be detected in the medium from transfected tunicamycin treated cells (Fig. 5, lane 3). This band had clearly a different mobility than the band of approximately 55 kDa that could be detected in the medium from transfected untreated cells (Fig. 5, lane 4). No immunoreactivity could be detected in the medium from non-transfected cells in the presence (Fig. 5, lane 5) or absence of tunicamycin (Fig. 5, lane 6). This strongly suggests that PAI-1 expressed in MDCK cells is *N*-glycosylated.

4. Discussion

Intensive research during the last three decades has focussed on determining the molecular basis for apical targeting of both secretory and transmembrane proteins. We have chosen to focus on *N*-glycan independent apical targeting of secretory proteins. The serpin family was chosen as model proteins because CBG, a member of the family, has been shown to be secreted mainly apically from MDCK cells [28] and because cDNA and antibodies are available to a large number of members of this family. This allows us to analyze the polarity of secretion of a number of serpins from MDCK cells and compare their primary sequences. Apart from *N*-glycosylation, which we have already shown is not important for the apical secretion of CBG [5], no other posttranslational modifications have been reported for serpins. This strongly indicates the presence of a hitherto unidentified proteinaceous apical sorting signal on members of this family.

It is still controversial whether *N*-glycans alone can act as an apical sorting signal. In the present study we have shown that PAI-1 is *N*-glycosylated and secreted in a non-sorted manner when expressed in MDCK cells [5]. This supports the hypothesis that *N*-glycans, instead of functioning as apical sorting signals, play a facilitative role that is necessary for the function of a proteinaceous apical sorting signal [4].

In the present study we expressed four members of the serpin family, α1-AT, C1-I, PAI-1 and AT, recombinantly in MDCK cells. The cells were grown to confluence on filters, the media collected and the amounts of serpin in the apical and basolateral compartments were analyzed. We found that α1-AT and C1-I were found mainly at the apical side, whereas PAI-1 and AT were distributed approximately equally between the two sides of the cells. In order to investigate whether this distribution reflects the polarity of secretion, we did a number of control experiments. We investigated the degree of transcytosis of the four serpins in the apical to basolateral direction and vice versa, the stability of PAI-I on both sides of the membrane and whether cells secreting PAI-1 and AT were able to secrete the endogenously expressed gp80 to the apical side. Our investigations showed that the four serpins are transported along the exocytotic pathway in an uncomplicated manner strongly indicating that the distribution between the apical and basolateral media reflects the polarization of the secretion.

Altogether three serpins, CBG [5,28], α1-AT and C1-I, are secreted mainly apically from MDCK cells whereas PAI-1 and AT are secreted in a non-sorted manner. A sequence comparison of these five serpins is shown in Fig. 6. Identical amino acids in the same position are indicated (bold). These residues are likely to play a role in forming and maintaining the three-dimensional structure of the molecule. Presumably an apical sorting signal was present in the ancestor protein of the serpin family and was lost in the evolution of PAI-1 and AT. In this case an apical sorting signal would be expected to be found in the three apically secreted serpins, and to be not found in the non-sorted serpins. There are 13 positions where the three apically secreted serpins have identical amino acids that are not present in either of the two non-sorted serpins (marked with an arrow in Fig. 6). None of these are adjacent to each other in the primary sequence. This suggests that the apical sorting signal does not consist of a continuous string of amino acids as does for example the KDEL signal that encodes retrieval to the endoplasmic reticulum [29]. It is, however, also possible that an apical sorting signal arose independently in the three apically targeted serpins. In that case the signal

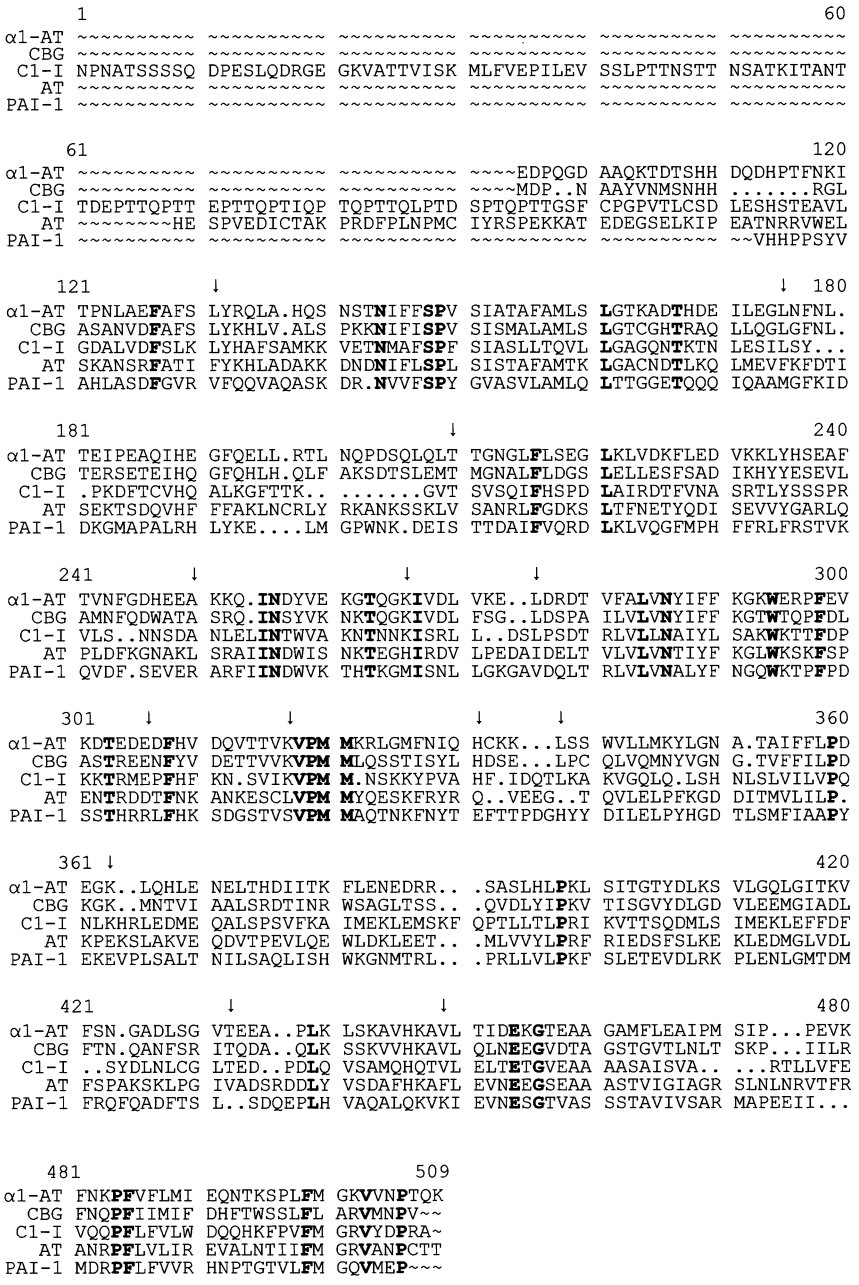

Fig. 6. Amino acid sequence alignments of human CBG, human α1-AT, human C1-I, human PAI-1 and rabbit AT. The alignment was performed with the Wisconsin Package, Version 9.1, Genetics Computer Group (GCG) using the program PileUp. Signal peptides are not shown. Conserved residues are in bold. Residues conserved between the three apically secreted serpins that are not present in any of the two non-sorted serpins are marked with arrows.

cannot be expected to be of similar structure and location. We find this possibility less likely.

Several attempts to identify apical sorting signals using chimeras between an apically and a non-sorted protein have failed because most of the chimeric proteins do not reach the cell surface. The serpin family may constitute a system that would allow chimeras between an apically and a non-sorted protein to reach the cell surface due to the homology in the amino acid sequence and their three-dimensional fold. The long-term goal of the present project is to identify the apical sorting signal on CBG. Based on the results obtained in the present study, we are currently investigating whether the apical sorting signal can be identified by generating chimeras between CBG and a non-sorted member of the serpin family followed by recombinant expression in MDCK cells and investigation of the polarity of expression.

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