Defective Sorting to Secretory Vesicles in Trans-Golgi Network Is Partly Responsible for Protein C Deficiency
Molecular Mechanisms of Impaired Secretion of Abnormal Protein C R169W, R352W, and G376D

Masao Naito, Jun Mimuro, Hitoshi Endo, Seiji Madoiwa, Kyo-ichi Ogata, Jiro Kikuchi, Teruko Sugo, Takanori Yasu, Yusei Kariya, Yuichi Hoshino, Yoichi Sakata

Abstract—Three thrombophilic patients with protein C (PC) deficiency were found to have independent mutations in the PC gene. These mutations resulted in single amino acid substitutions of R169W, R352W, and G376D in the affected PC molecules. These abnormal PC molecules were expressed in CHO-K1 cells in the presence or absence of vitamin K, and their synthesis, posttranslational modification, and secretion were studied. PC G376D was not secreted from the cells and was gradually degraded inside the cells. There was partial secretion of PC R169W and PC R352W, but most of these molecules were not secreted but were degraded intracellularly. On the basis of pulse-chase, immunofluorescence, and endo-β-N-acetylglucosaminidase H digestion experiments, the majority of wild-type PC molecules localize not in the Golgi apparatus but in the rough endoplasmic reticulum inside the cells. This suggests that wild-type PC molecules are secreted immediately after \( \gamma \)-carboxylation and modification at the Golgi apparatus. In contrast, the mutant PC molecules were retained inside the cells even after modification of oligosaccharides at the trans-Golgi apparatus, which was probably due to impaired conformation of the abnormal molecules. Data suggest that these abnormal PC molecules were not sorted to secretory vesicles in the trans-Golgi network because of conformational defects in addition to the transport defect from the rough endoplasmic reticulum to the Golgi apparatus and were degraded inside the cells, thereby resulting in a PC deficiency in the affected patients. (Circ Res. 2003;92:865-872.)

Key Words: thrombosis ■ protein C deficiency ■ intracellular protein transport

The protein C (PC) system is an important regulatory mechanism of blood coagulation, without which the integrity of the vascular system is perturbed by intravascular coagulation.1 PC, a member of the vitamin K–dependent protein family, is synthesized in the liver and is secreted into the circulation as a zymogen, analogous to the vitamin K–dependent coagulation factors.1–3 PC degrades the cofactors of blood coagulation, factors Va and VIIIa, on activation to activated PC by thrombomodulin-bound thrombin.1 Human PC is synthesized as a 461-amino-acid single polypeptide chain that undergoes cotranslational and posttranslational modification, including glycosylation, \( \gamma \)-carboxylation, \( \beta \)-hydroxylation, propeptide cleavage, and conversion to the two-chain form.1,4,5 Human PC has four N-linked glycosylation sites, three of which are located in the heavy chain.4,6 Glycosylation at the light chain site is shown to be required for efficient secretion.1,6 Specific Glu residues that reside in the NH\(_2\)-terminal region of the light chain are \( \gamma \)-carboxylated by vitamin K–dependent \( \gamma \)-carboxylase,7–9 giving rise to this domain being designated the Gla domain. The \( \gamma \)-carboxyglutamic acid residues of PC are required for anticoagulant function. In the human PC, Asp71 is modified to a \( \beta \)-hydroxylated residue and is thought to be involved in high-affinity calcium ion binding.10

Many clinical reports have shown that patients with decreased circulating levels of PC develop thrombophilia,11–15 with venous thrombosis in heterozygotes and purpura fulminans in homozygotes. A wide variety of genetic mutations can lead to PC deficiency and PC molecular abnormality.11–15 We found genetic mutations in three patients with thrombotic episodes and decreased levels of plasma PC. In the present study, we expressed wild-type PC and PC mutants corresponding to the respective genetic abnormalities of each patient to elucidate the molecular mechanisms of the secretion of wild-type PC and impaired secretion of abnormal PC.
Materials and Methods

Determination of Nucleotide Sequences of the Patient PC Gene

Genomic DNA was isolated from peripheral leukocytes by standard procedures as described. 16 DNA fragments spanning all the exons including the exon-intron boundaries of the PC gene were amplified by polymerase chain reaction using Pyrobest DNA polymerase (Takara Shuzo). The nucleotide sequences of the amplified DNA fragments were determined using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and DNA sequencer model ABI 310 (Applied Biosystems Inc).

Expression of Human PC in Cultured Cells

cDNA for human PC was a generous gift from Dr S. Hiroswa (Tokyo Medical and Dental University, Tokyo, Japan). 17 The human PC cDNA spanning the entire coding region was cloned downstream from the cytomegalovirus promoter of plasmid pcDNA3 (Invitrogen, Corp) in the appropriate orientation to make plasmid pcDNA/hPCWT, pcDNA/hPCR169W, or pcDNA/hPCG376D. These constructs were used for expression of PC R169W, PC R352W, and PC G376D in mammalian cells.

Antibodies, ELISA, and Western Blotting

Monoclonal antibodies used in the present study have been described previously. 18,19 Antibodies JTC1 and JTC3 recognize the calcium ion–dependent conformation of the Gla domain of human PC. 19 Antibody JTC3 has been shown previously not to bind to PC R169W, a mutant PC with amino acid substitution R15G in the Gla domain. 16 Antibody JTC4 binds to the catalytic domain of PC, and its binding to the PC catalytic domain is decreased significantly after reduction. Antibody JTC5 binds to the activation peptide. 18 Polyclonal antibodies to human PC were purchased from DAKO Japan and labeled with biotin using N-hydroxysuccinimido-biotin. For ELISA, plastic microtiter plates were coated with either the polyclonal antibody or a monoclonal antibody in PBS at 4°C for 16 hours. After blocking with 5% casein in PBS, microtiter plates were incubated with PC-containing samples in ELISA buffer (50 mmol/L Tris, 0.15 mol/L NaCl, 5 mmol/L CaCl2, 1% casein, and 0.1% Triton X-100) at 37°C for 2 hours. Microtiter plates were washed and incubated with biotinylated polyclonal antibody, followed by streptavidin-labeled horseradish peroxidase and substrate. The half-maximum binding concentration of PC mutants to monoclonal antibody JTC4 was determined by incubation of increasing concentrations of cell extract PC with the monoclonal antibody–immobilized microtiter plates, followed by detection of monoclonal antibody–bound PCs by biotin-labeled polyclonal antibody, as described above. Western blotting analysis for human PC was performed as described previously except for detection of membrane-bound horseradish peroxidase–labeled antibody using chemiluminescence reagent ECL Plus (Amersham Pharmacia Biotech). The PC used as a standard for the ELISA and the control PC sample for the Western blotting studies were purified from human plasma as described. 20

Expression of Recombinant PC In Vitro

CHO-K1 cells were cultivated in HAM-F12 medium in the absence of vitamin K. CHO-K1 cells (5x106) in 0.8 mL Dulbecco’s PBS were incubated with 20 µg of pcDNA/hPCWT, pcDNA/hPCR169W, pcDNA/hPCR352W, or pcDNA/hPCG376D for 30 minutes at 4°C and subjected to electroporation at 300 V (25 µF) using a Gene Pulsor (Bio-Rad Laboratories). To make stable cell lines that express wild-type PC and the three PC mutants, cells were cultured in the presence of Geneticin (250 µg/mL, GIBCO-Invitrogen Japan). Transfected cells were subjected to electroporation with pcDNA3 alone, were also established for the control. Cloned CHO-K1 cells expressing wild-type PC and mutant PC molecules were cultured in the presence or absence of vitamin K (10 µg/mL). Expression of PC in the conditioned medium and in cell extracts in PBS containing Triton X-100 (0.5%) and phenylmethylsulfonyl fluoride (1 mmol/L) was analyzed by ELISA and Western blotting.

Analysis of Posttranslational Modification and Secretion of PC and PC Mutants

Cells were cultured for 1 hour in methionine-deficient medium (GIBCO-Invitrogen Japan) containing 100 µCi/mL [35S]methionine (NEL Life Science Products, Inc), washed with PBS, and then cultured in HAM-F12 supplemented with vitamin K (10 µg/mL). After various incubation periods, conditioned medium and cell extracts were prepared. 16 Radio-labeled PC molecules in conditioned medium and cell extracts were subjected to immunoprecipitation using polyclonal antibody against PC and protein A–coupled Sepharose CL-4B (Amersham Pharmacia Biotech). [35S]-labeled PC was analyzed by SDS-PAGE, followed by autoradiography, and was quantified using an image analyzer (LAS 2000, Fujifilm).

Cellular Localization of Wild-Type PC and PC Mutants

Cloned cells expressing wild-type PC and PC mutants were seeded on a glass-bottom dish coated with poly-L-lysine (Matsumi Glass Ind, Ltd). After a washing with the HBSS-HEPES buffer (Hanks’ buffered salt solution containing 10 mmol/L HEPES, pH 7.4) was performed, the cells were incubated for 30 minutes at 4°C with 5 µmol/L BODIPY-TR ceramide (Molecular Probes, Inc), a fluorescent sphingolipid. Cells were then incubated in fresh medium at 37°C for 30 minutes more. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes and then permeabilized with 0.05% Triton X-100 for 10 minutes at room temperature. After 16 hours of incubation with 1 µg/mL rabbit anti-human PC antibody in PBS at 4°C, the cells were incubated with Alexa 488–labeled goat anti-rabbit IgG (H+L, Molecular Probes, Inc) for another 1 hour at room temperature. Cells were visualized using a confocal laser microscope system μ Radiance (Bio-Rad Laboratories) and ECLIPSE TE 300 (Nikon). The images (PC, green; Golgi apparatus, red) were merged for evaluation of localization of PC in the Golgi apparatus (yellow), and yellow areas and green areas (intracellular PC) were quantified using image analysis software (Mac Scope, version 2.5, Mitani Co).

Analysis of Glycosylation of Wild-Type PC and PC Mutants

Wild-type PC and mutant PC were isolated from the conditioned medium, and the extracts of cells were incubated with [35S]methionine in the presence of vitamin K (10 µg/mL) or warfarin (1 µmol/L) using anti-PC polyclonal antibody and protein A–coupled Sepharose CL 4B as described above. [35S]Methionine-labeled PC was then incubated with 0.1 U/mL endo-β-N-acetylglucosaminidase H (Endo H) in PBS, pH 6.0, in the presence of 0.1% SDS at 37°C for 16 hours. Samples were analyzed by SDS-PAGE, followed by autoradiography.

Results

Genetic Abnormalities of Patients With Decreased Levels of Plasma PC

Analysis of the PC genes of the three thrombotic patients with decreased plasma levels of PC was performed. After sequencing all exons and exon-intron boundaries, we found independent heterozygous single nucleotide mutations of C to T at position 6268, C to T at position 8769, and G to A at position 8842 in the affected PC genes. These genetic mutations resulted in single amino acid substitution of R169W, R352W, and G376D of the respective PC molecule.
These genetic abnormalities were the same mutations reported previously, but PC R352W and PC G376D had not been observed previously in the Japanese population. PC R169W has a molecular defect in the activation process by the thrombin/thrombomodulin complex, and PC plasma levels are decreased, although the abnormal molecules are present in the circulation. Patients with mutants PC R352W and PC G376D reportedly develop a type I PC deficiency, but the molecular basis for the impaired secretion of these abnormal molecules has not been reported.

Recognition of Recombinant PC Antigens in Cultured Cells by Monoclonal Antibodies
To elucidate molecular mechanisms of PC deficiency caused by these genetic and molecular abnormalities, stable CHO-K1 cell lines expressing wild-type PC, PC R169W, PC R352W, and PC G376D were created. Polyclonal or monoclonal anti-PC antibodies were used as capture antibodies in ELISAs to quantify PC antigen levels in conditioned media (Table) and in cell extracts (Figure 1). Comparison of the total antigen levels (polyclonal antibody) with binding by the monoclonal antibodies permits identification of PC molecules with altered conformation of the Gla domain (JTC1 and JTC3), catalytic domain (JTC4), or activation peptide region (JTC5). When CHO-K1 cells expressing PC were cultured in the presence of vitamin K, the level of wild-type PC in the conditioned media determined by JTC1 or JTC3 ELISA was similar to that determined by polyclonal ELISA (Table), suggesting that wild-type PC expressed in CHO-K1 cells was γ-carboxylated with proper Gla domain conformation and that CHO-K1 cells were suitable for studying the synthesis and secretion of γ-carboxylated proteins. The levels of PC R169W and PC R352W in the conditioned media determined by JTC1 or JTC3 ELISA were similar to the levels determined by polyclonal ELISA, again suggesting that the PC R169W and PC R352W were fully γ-carboxylated, although the absolute amounts of these PC mutants secreted into the conditioned medium were lower than those with wild-type PC.

In the cell extracts, wild-type PC was recognized similarly by the anti-PC polyclonal antibody and the JTC4 monoclonal antibody that binds to the serine proteinase domain (Figure 1C). In contrast, binding levels relative to the polyclonal antibody binding were significantly reduced when they were assayed using the JTC1 (19%) or JTC3 (2%) monoclonal antibodies, which recognize conformational epitopes in the Gla domain (Figures 1A and 1B). Western blotting analyses confirmed ELISA data (not shown). PC R169W and PC R352W antigen in the cell extracts also bound similarly to JTC4 and the anti-PC polyclonal antibodies, but binding was reduced to 10% to 15% on a JTC1 surface, and there was no binding at all to JTC3. Binding of PC G376D to JTC1 and JTC3 was similar to the other two mutant PC molecules from cell extracts. These data suggested that the majority of the wild-type PC and mutant PC in the cell extracts did not have a Gla domain conformation analogous to native PC.

<table>
<thead>
<tr>
<th>Concentration of PC in the Conditioned Medium of CHO-K1 Cells</th>
<th>Polyclonal ELISA, ng/mL (%)</th>
<th>JTC1 ELISA, Percentage of Polyclonal ELISA</th>
<th>JTC3 ELISA, Percentage of Polyclonal ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>702±5.10 (100%)</td>
<td>91.7±6.25</td>
<td>92.5±2.84</td>
</tr>
<tr>
<td>R169W</td>
<td>54.4±7.47 (100%)</td>
<td>83.3±8.44</td>
<td>91.1±7.96</td>
</tr>
<tr>
<td>R352W</td>
<td>59.1±5.33 (100%)</td>
<td>90.1±8.00</td>
<td>84.7±8.81</td>
</tr>
<tr>
<td>G376D</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Concentrations of PC in the conditioned medium of CHO-K1 cells, which express wild-type PC, PC R169W, PC R352W, or PC G376D in the presence of vitamin K, were determined by polyclonal ELISA, JTC1 ELISA, or JTC3 ELISA as described in Materials and Methods. The amounts of PC determined by JTC1 ELISA or JTC3 ELISA were expressed as the percentages of that determined by polyclonal ELISA (mean±SD, n=4).

Figure 1. Analysis of recombinant PC in the cultured cells by monoclonal antibodies. Recombinant PC molecules (wild-type PC, R169W, R352W, and G376D) expressed in the cell extracts of stably transfected CHO-K1 cells were quantified by ELISA using either polyclonal antibody or monoclonal antibodies, JTC1 (A), JTC3 (B), JTC4 (C), or JTC5 (D) as a capture antibody as described in Materials and Methods. The amount of PC antigen determined using the monoclonal antibody-based assays was expressed as the percentage of that determined by the polyclonal ELISA. *P<0.01 (n=4).
binding of PC R169W and PC R352W from cell extracts to JTC4 antibody was similar to that of wild-type PC, suggesting that the serine proteinase domain structure was relatively less affected and that the conformational changes were not global. However, binding of the PC G376D mutant from the cell extracts to JTC4 was significantly decreased relative to wild-type PC or the other mutants, indicating structural changes in the proteinase domain for this mutant. Additional observations were that the relative PC G376D concentration determined by JTC5 ELISA was ~3-fold higher than that of wild-type PC determined by JTC5 ELISA (Figure 1D) and that the half-maximum binding of PC G376D to JTC5 was achieved at 50 ng/mL, whereas the half-maximum binding of wild-type PC to JTC5 was obtained at 230 ng/mL (not shown), suggesting that the conformational changes that occurred in the PC G376D mutant allowed JTC5 to access to the activation peptide of the PC molecule with the higher affinity. Binding of PC R352W to the JTC5 antibody was similar to that observed for wild-type PC, but binding of PC R169W was reduced ~80%. This decrease may be accounted for by the fact that PC R169W has an amino acid substitution in the activation peptide, which probably alters the epitope for this monoclonal antibody.

Synthesis and Secretion Rates of Wild-Type PC and PC Mutants

We performed pulse-chase experiments to study the synthesis and secretion of wild-type PC and the mutants. Wild-type PC was secreted gradually from CHO-K1 cells in the presence of vitamin K during the chase period (Figure 2A). Wild-type PC in the cell extracts decreased, and the total amount of [35S]methionine-labeled wild-type PC (the sum of secreted PC and the PC in the cell extracts) did not change. PC R169W and PC R352W in the cell extracts decreased faster than did wild-type PC, but only 10% of the PC molecules were secreted into the conditioned medium (Figures 2B and 2C). The total amount of [35S]methionine-labeled PC decreased during the incubation period, suggesting that most of the [35S]methionine-labeled PC was degraded inside the cells. PC G376D was not secreted into the conditioned medium at all,
but the rate of decrease inside the cells was slightly slower in PC G376D than in PC R169W and PC R352W (Figure 2D). This may be explained by the observation that PC G376D was not secreted at all and suggests that the rates of degradation of these mutant molecules were similar. Collectively, the overall data demonstrate that wild-type PC is secreted from cells immediately after \(/H9253/-carboxylation and modification at the Golgi apparatus without degradation, whereas secretion of PC R169W, PC R352W, and PC G376D is severely or completely impaired, and the molecules are degraded inside the cells.

**Cellular Localization of Wild-Type PC and PC Mutants**

To study the localization of wild-type PC and the mutant PC in the cells, immunofluorescence colocalization studies were performed using two-color confocal microscopy. The transfected cells were incubated with BODIPY-TR ceramide, a fluorescent sphingolipid that is incorporated into the Golgi apparatus. The cells were then processed for PC antigen using anti-PC polyclonal antibody and Alexa-488–labeled anti-goat IgG. As shown in Figure 3, the majority of wild-type PC did not colocalize with the fluorescent signal in the Golgi apparatus, suggesting that the transit time in the Golgi for wild-type PC is fairly short. PC R169W and PC R352W appear to colocalize with the Golgi more than does wild-type PC, suggesting that these mutant PC molecules might be retained in the Golgi apparatus. PC G376D was not secreted from the cells at all, but this abnormal molecule also colocalized with the Golgi, suggesting that a certain amount of PC G376D was transported to the Golgi.

**Glycosylation of Wild-Type PC and PC Mutants**

\(^{[35]}S\)Methionine-labeled wild-type and PC mutants in the conditioned medium and in the cell extracts synthesized in the presence or absence of vitamin K were isolated by immunoprecipitation and treated with Endo H. Endo H hydrolyzes Asn-linked oligosaccharides with a mannose core.\(^{22,23}\) However, Endo H is unable to hydrolyze complex oligosaccharides of glycoproteins once a modification, such as addition of sialic acid, takes place in the trans face of the Golgi apparatus. Thus, the resistance of oligosaccharide side chains to Endo H is thought to be a marker of the posttranslational processing of the glycoprotein in the trans face of the Golgi.\(^{22,23}\) As expected, oligosaccharides of wild-type and mutant PC in the conditioned medium were resistant to Endo H (Figure 4), suggesting that their carbohydrate side chains were complex oligosaccharides. In the presence of warfarin, less wild-type PC was secreted into the media, but their oligosaccharides were Endo H resistant, suggesting that \(/H9253/-carboxylation was not mandatory for modification of oligosaccharides in the Golgi apparatus. PC R169W and PC R352W were partly secreted in the presence of vitamin K but were not secreted at all in the presence of warfarin. The majority of wild-type PC in the cell extracts was sensitive to Endo H and was converted to the deglycosylated form (indicated by an asterisk) by Endo H treatment. Thus, the majority of wild-type PC found in the cell extracts did not undergo modification of carbohydrate side chains at the trans face of the Golgi apparatus. The presence of vitamin K or warfarin did not affect the susceptibility of oligosaccharide side chains of wild-type PC in the cell extracts to Endo H. However, the amount of PC secreted into the conditioned medium in the presence of warfarin was less than that in the presence of vitamin K. When cells were cultured in the presence of vitamin K, PC R169W and PC R352W in the cell extracts were converted to the deglycosylated form by Endo H, and populations that migrated slower than the deglycosylated form of wild-type PC were observed (double asterisk), indicating that this population of PC R169W and PC R352W

![Figure 3. Cellular localization of wild-type PC and PC mutants. Cells expressing wild-type PC and mutant PC (R169W, R352W, or G376D) were incubated in the presence of fluorescently labeled sphingolipids (red), followed by immunofluorescence detection of PC antigen (green) using a confocal laser microscopy as described in Materials and Methods. Mock-transfected cells (Mock) were processed simultaneously as the control. The images were merged for evaluation of regions of colocalization (yellow). After quantitative analysis, the yellow area (PC colocalized with the Golgi apparatus) was expressed as the percentage of the green area (intracellular PC) and was shown in the right side of images (PC in the Golgi apparatus: mean±SD, n=4). *P<0.01 for differences between areas of colocalization. ND indicates not determined.

![Image of Figure 3](http://circres.ahajournals.org/Downloaded image)
had undergone modification of oligosaccharide side chains in the trans face of the Golgi apparatus. In the presence of warfarin, PC R169W and PC R352W in the cell extracts were converted to the deglycosylated form and were not secreted into the conditioned medium. Similarly, PC G376D in the cell extracts had complex oligosaccharide side chains when cells were cultured in the presence of vitamin K. Taken together, these data support the notion that wild-type PC is secreted rapidly from cells on modification of the oligosaccharide side chain in the Golgi apparatus. In contrast, the PC mutants are retained inside the cells, even after modification of oligosaccharide side chains in the trans face of Golgi apparatus, and γ-carboxylation of the Gla domain of PC is necessary for efficient transport of PC from the endoplasmic reticulum (ER) to the Golgi apparatus.

**Discussion**

A variety of genetic abnormalities are responsible for PC deficiency (type I) and abnormalities (type II) associated with thrombotic episodes, and many reports have shown that even single amino acid substitutions can cause a type I PC deficiency.11–15 On the basis of analyses of synthesis rates, posttranslational modifications, and secretion of wild-type PC and mutant PC with single amino acid substitution, we demonstrated that conformational defects resulted in the impaired sorting of abnormal molecules to secretory vesicles in the trans-Golgi network. This impairment may be at least partly responsible for PC deficiency caused by single amino acid substitution.

Because the majority of wild-type PC in the cell extracts did not bind to JTC3 monoclonal antibody and did not localize with the Golgi apparatus and because the oligosaccharides were sensitive to Endo H, wild-type PC appeared to reside mainly in the rough endoplasmic reticulum (RER) and was secreted quickly once it underwent modification at the Golgi, as reported previously.24 According to the ELISA and pulse-chase data, secretion of the three PC mutants was impaired. Only small amounts of PC R169W and PC R352W were secreted, and PC G376D was not secreted at all from the cells. In contrast to wild-type PC, a portion of the PC mutants was retained inside the cells even after receiving oligosaccharide side chain modification at the Golgi apparatus. The observation that PC R169W and PC R352W colocalized with the Golgi to a greater extent than did wild-type PC also supports this notion. Data indicate that binding of PC G376D to JTC4 (epitope: catalytic domain) is decreased, whereas binding to JTC5 (epitope: activation peptide) is increased, supporting the notion that the conformation of the G376D mutant, in which secretion is completely abolished, is perturbed more severely than it is for the two other mutants. Taken together, these data indicate that an abnormal conformation induced in the catalytic domain of PC, together with an additional conformational change of the Gla domain, results in a secretion defect of mutant PC molecules to secretory vesicles in the trans-Golgi network.

Intracellular protein transport is a complex process coordinated by a variety of molecules. Compared with protein transport from RER to the Golgi apparatus by the ER quality control machinery, the regulatory mechanisms of protein transport from the Golgi apparatus to secretory vesicles and to cell surfaces are not well understood. Recently, the presence of sorting receptors and their important roles in protein transport from the Golgi have been proposed.25,26 An association of secretory proteins with sorting receptors and/or trans-Golgi network membranes may be important in proteins for secretion.27 Thus, a transport defect in abnormal molecules could reside in the trans-Golgi network. Impaired transport of an abnormal PC from the RER to the Golgi apparatus was proposed for the type I deficiency that is due to an abnormal PC molecule that has a substitution of the 39 carboxyl-terminal amino acids with 81 meaningless residues caused by a frame-shift mutation.24 Although it was difficult to quantify PC molecules at each transport step in the present

![Figure 4. Analysis of carbohydrate side chains of wild-type PC and mutant PC. Stably transfected CHO-K1 cells expressing wild-type PC and PC mutants were incubated in medium containing [35S]methionine for 1 hour, washed, and incubated in the standard medium in the presence of vitamin K (Vit K) or warfarin (Wa) as described in Materials and Methods. PC molecules in the cell extracts obtained after 1 hour of labeling and those in the conditioned medium, harvested after 8 hours of incubation, were isolated by immunoprecipitation. After dissociation of PC from Sepharose-CL4B by heating in the presence of SDS, samples were incubated in the presence or absence of 0.1 U/mL Endo H for 16 hours. These samples were analyzed by SDS-PAGE, followed by autoradiography. *Deglycosylated PC. **Endo H-resistant PC.](http://circres.ahajournals.org/doi/10.1161/01.RES.870.9.1518)
study, decreased transport of PC R169W, PC R352W, and PC G376D mutants from the RER to the Golgi apparatus by the ER quality control mechanism might partly account for the impaired secretion of these molecules because these abnormal molecules appeared to have a conformational defect(s). The Endo H digestion study also clearly showed that Endo H–resistant abnormal molecules resided in the cell extracts, suggesting that retention of abnormal molecules in the Golgi apparatus did exist. In addition, data indicating that PC G376D, which was not secreted from the cells at all, was colocalized with the Golgi apparatus suggested that this abnormal molecule was transported from the RER to the Golgi and then retained. Thus, defective sorting to secretory vesicles in the trans-Golgi network would also be partly responsible for the patient with PC deficiency. To our knowledge, this is the first demonstration of a defect in the transport of a vitamin K–dependent protein from the Golgi apparatus to secretory vesicles.

In addition to binding to a sorting receptor for regulated secretory proteins, association of proteins to the membrane of the trans-Golgi network is thought to be necessary for the segregation of proteins to secretory vesicles. In that context, the difference in reactivity to the JTC3 monoclonal antibody between the mutant PC molecules in the cell extracts and in the conditioned medium was interesting. PC R169W and PC R352W were not secreted well, but the majority of those PC molecules that were secreted were bound to JTC3. However, PC R169W and PC R352W in the cell extracts did not bind to JTC3 at all, even though certain amounts of these molecules were transported to the trans-Golgi apparatus.

Like other vitamin K–dependent coagulation factors, PC undergoes posttranslational processing. As well as N-linked glycosylation, γ-carboxylation of vitamin K–dependent proteins is thought to take place before transport to the Golgi apparatus and possibly as a cotranslation event in the RER. Because the PC mutants in the present study have an amino acid substitution in the catalytic domain, cotranslational γ-carboxylation of the Gla domain of these molecules is likely to be processed properly. Inasmuch as mutant PC in the cell extracts did not bind to JTC3 antibody, the amino acid substitutions in the catalytic domain may have affected the trans-Golgi apparatus and possibly as a cotranslation event in the RER. The presence of vitamin K. However, these mutant molecules in the cell extracts were sensitive to Endo H, and no mutant PC molecules were present in the conditioned medium when the cells were cultured in the presence of warfarin. These data suggest that γ-carboxylation of PC is necessary for the efficient transport of PC from the ER to the Golgi apparatus but that it is not absolutely required for secretion from the cells. It is well known that vitamin K antagonist treatment causes not only secretion of dysfunctional vitamin K–dependent coagulation factors but also decreased plasma levels of these molecules, although the mechanism for the decreased plasma levels are not known. In accordance with previous studies, data shown in the present study indicate that the primary cause of decreased secretion of vitamin K–dependent coagulation factors from the cells during vitamin K antagonist treatment is inefficient transport of these molecules from the RER to the Golgi apparatus.

In conclusion, an appropriate conformation of PC and other vitamin K–dependent factors is required for efficient transport from the RER to the Golgi apparatus and from the trans-Golgi apparatus to secretory vesicles. A conformational defect in the catalytic domain caused by single amino acid substitutions results in a type I deficiency, which is partly due to defective sorting of the abnormal molecules into secretory vesicles at the trans-Golgi network.

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References


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