Glycosylation of Human Protein C Affects Its Secretion, Processing, Functional Activities, and Activation by Thrombin*

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Human protein C (HPC) is an antithrombotic serine protease that circulates in the plasma as several glycoforms. To examine the role of glycosylation in the function of this protein, we singly eliminated each of the four potential N-linked glycosylation sites by site-directed mutagenesis of HPC or to Gln at amino acid positions 97, 248, and 313 (HPC derivatives Q97, Q248, and Q313) or at the unusual consensus sequence Asn-X-Cys at 329 (HPC derivative Q329). The cDNAs for wild type and each derivative were inserted into expression vectors and expressed both transiently and stably in human 293 and hamster AV12-664 cells. We demonstrate that N-linked glycosylation at position 97 in the light chain of HPC is critical for efficient secretion and affects the degree of core glycosylation at Asn-239. Glycosylation at position 248 affects the internal cleavage site, and partial glycosylation at the sequence Asn-329-X-Cys is responsible for the natural α-glycoform. Altering the glycosylation pattern of the protein had no significant effect on the level of fully γ-carboxylated HPC secreted from the 293 cell line. However, elimination of glycosylation sites in the heavy chain resulted in a 2- to 3-fold increase in anticoagulant activity. Utilizing synthetic substrate, both the $k_{cat}$ and $K_m$ were affected, depending on the specific glycosylation site eliminated. However, there were no significant differences in the inhibition kinetics by α-1-antitrypsin (association rate constants of 10–11 M⁻¹ s⁻¹ and $t_{1/2}$ of 27–29 min at 40 μM α-1-antitrypsin) or $t_{1/2}$ in human plasma (17–18 min). A comparison of the rate of activation of each derivative by thrombin alone or in complex with thrombomodulin revealed that Q313 was activated ~2.5-fold faster than wt HPC, independent of calcium concentration. This increase in rate was due to an enhanced affinity of thrombin-thrombomodulin for the modified Q313, as indicated by a 3-fold reduction in $K_m$. Overall, our studies demonstrate that glycosylation at different sites in HPC affects distinct properties of this complex protein. Furthermore, we demonstrate the ability to improve the catalytic efficiency of this enzyme through carbohydrate modifications.

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Human protein C is a vitamin K-dependent plasma glycoprotein that circulates as an inactive zymogen. At the endothelial cell surface, it is converted to its active form by limited proteolysis with α-thrombin in complex with a cell surface membrane protein, thrombomodulin (1–3). The activated form of protein C (αPC) has potent anticoagulant activity due to its ability to inactivate factors Va and VIIIa (4), and it has been reported to have profibrinolytic activity (5, 6). Protein C plays a critical role in the regulation of thrombin generation (reviewed in Refs. 7–9) and may be effective in the treatment of a number of thrombotic diseases (7, 10).

The native HPC molecule is shown schematically in Fig. 1. HPC circulates predominantly as a disulfide-linked heterodimer (7, 11) composed of a light chain ($M_r \sim 25,000$) and a heavy chain ($M_r \sim 41,000$) containing the serine protease domain. The remaining material circulates in a single chain form, lacking the removal of an internal Lys-Arg (KR) dipeptide (12). The light chain contains the region of γ-carboxyglutamic acid (Gla) residues that is highly conserved among vitamin K-dependent proteins. This specialized post-translational modification is required for calcium-dependent membrane binding and functional activity (reviewed in Ref. 8). In addition to this complex modification, HPC contains ~1 residue of β-hydroxyaspartic acid (Asp-71) (13–15) that is believed to be involved in a Gla region-indepedent calcium-binding site (16, 17).

Both plasma-derived and recombinant HPC have several forms of the heavy chain, designated α, β, and γ, with apparent $M_r$ of ~41,000, 37,000, and 32,000, respectively. Previously, we demonstrated that recombinant HPC secreted from tunicamycin-treated cells (18) contained one heavy chain band, which also was observed by Yan et al. (15) following treatment of purified HPC with N-glycanase. These data suggested that the various subforms represented differences in glycosylation pattern. Of the four potential sites for N-linked glycosylation in HPC, one is in the EGF domain of the light chain at amino acid position 97, and the remaining three are in the heavy chain at positions 248, 313, and 329. Interestingly, the site at 329 contains the unusual sequence Asn-X-Cys that has been shown to be glycosylated in bovine protein C (19) and in von Willebrand Factor (20). In this paper, we examine the role of glycosylation at each of the four potential sites in HPC. We demonstrate that glycosylation of HPC at specific sites influences the secretion, proteolytic processing, and rate of activation by thrombin.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs, mutagenesis was performed with QuikChange™ site-directed mutagenesis kit (Stratagene), restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs, and recombinant human protein C was from Calbiochem. Human plasma (17–18 min).

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performed using Bio-Rad Mutazyme in vitro mutagenesis kit (catalog #170-3571). agarose was from Bethesda Research Laboratories, and chemicals were purchased from Boehringer Mannheim and/or Sigma. Ham’s F-12 and Dulbecco's modified Eagle's medium were purchased from Gibco, and fetal bovine serum was from HyClone. Eli Lilly supplied bovine insulin and hygromycin B. Hirudin (H-7016) and chemicals were purchased from Boehringer Mannheim and/or Sigma. Hirudin (#170-3571) and agarose was from Bethesda Research Laboratories, and chemicals were purchased from Miles Scientific. Rabbit thrombomodulin was obtained from Esmon (Oklahoma Medical Research Foundation) or was purchased from American Diagnostica. The cDNA for the major late promoter was a gift from Dr. J. Rose of the Biotechnology Laboratory (BRL) containing the enhancer region of human adenovirus type 2 (25). The first EGF domain through approximately half of the serine protease domain was isolated from the plasmid pLPC (13) and cloned into both M13mp18 and M13mp19. These clones, designated MP18-nHPC.730 and MP18-nHPC.730, were ultimately used as the source of single-stranded DNA template for generation of glycosylation mutants MP18-Q313 and MP18-Q329. Oligonucleotide primers were made with an Applied Biosystems model 380 DNA synthesizer and had the following sequences: Pm-Q097, 5'-GGAGGCATGAGCATGTCGCA-3'; Pm-Q248, 5'-CCGGTGTGTCGCAATGTGC-3'; Pm-Q313, 5'-CCGGAGAGTAGAAGCAGCACCGAG-3'; Pm-Q329, 5'-GATCCCTGTTGCGGCAAGCT-3'. These primers were designed not only to change the codon of their corresponding asparagine residue for use as template. Approximately 250 ng of template was annealed to 4 pmol phosphorylated primer in 10 μl of buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl2, and 50 mM NaCl at 85 °C, allowed to slow-cool to room temperature over a period of approximately 2 h, and then placed on ice. The primer extension reaction was modified as follows: initial incubation was for 15 min at 4 °C, 15 min at 25 °C, and 90 min at 37 °C, followed by the addition of 5 μl of deionized/ distilled water, 1 μl of ligation buffer (10 x as described by New England Biolabs), and 1 μl of T4 DNA ligase (400 units/μl) for a final volume of 20 μl. Incubation was continued overnight at 16 °C, and then 80 μl of deionized/distilled water was added as diluent, and the reaction was frozen briefly at −20 °C. Competent MV1190 (doT+) and BsmI (Q248) were transformed into competent CJ236 (doT- ung-), and restriction enzyme digestion of the major late promoter, a synthetic sequence comprising the spliced leader sequence and the entire protein C insert was isolated. Reconstitution of the complete protein C coding sequence was accomplished via a three-part ligation involving the 3788-base-pair EcoRI/Sall fragment from pLPC, a 730-base-pair Sall/Scl fragment from pLPC, MP18-Q097, or MP19-Q248 and a 2018-base-pair Sall/EcoRI fragment (from pLPC, MP18-Q313, or MP18-Q329). DNA from the resultant clones (designated pLPC-Q097, pLPC-Q248, pLPC-Q313, and pLPC-Q329) was transformed into Escherichia coli strain GM48 (dam–) to facilitate removal of the entire protein C coding sequence on a BclI fragment. Glycosylation of Recombinant Protein C—The 1425-base-pair BclI fragment of each of the pLPC clones above was excised and inserted into the eukaryotic expression vector pGT-hyg at its unique BclI site. Plasmid pGT-h derivatives contain the following elements: the Eucaryotic leader sequence from human adenovirus type 2. HindIII and BclI linker, the Bantl to SphI fragment of the cDNA coding sequence for human protein C (21) flanked by BclI linkers, the 610-bp MboI fragment of SV40 containing the small t splice junction, and the 988-base-pair BclI to EcoRI fragment of SV40 containing the polyadenylated transcriptional termination signal. The completed expression plasmid was designated pGTH-Q097, pGTH-Q248, pGTH-Q313, and pGTH-Q329, where Qn designates the Asn to Gln substitution at the indicated amino acid position (n). Each expression plasmid was purified on a CsCl density gradient, linearized with FspI, and used to transfect 293 cells.

Cell Culture, DNA Transfection, and Drug Selection—The adenovirus-transformed human kidney 293 (ATCC CRL 1573) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, and 10 μg/ml vitamin K1. One day prior to transfection, cells were plated at a density of 106 cells/ 5 cm2. Calcium phosphate-DNA precipitates were prepared (25) with 10 μg of expression plasmid DNA and no additional calcium phosphate. Four hours after transfection, the culture medium was replaced, and 2-3 days after transfection the culture medium was replaced with selection medium containing 200 μg of hygromycin B/ml. Clonal cell lines resistant to hygromycin B were isolated 4-5 weeks after applying drug selection. These recombinant cell lines were expanded, and the presence of HPC in the conditioned culture medium was determined by the enzyme-linked immunosorbent assay described below.

Detection of HPC Antibodies—A solid phase sandwich enzyme-linked immunosorbent assay, with a sensitivity of 5 ng/ml, was used to measure the level of HPC-specific antibodies in the serum of rats. 96-well plates were coated either with sheep anti-HPC polyclonal antibody or a murine monoclonal antibody. HPC antibody sandwiched between the polyclonal antibodies was followed by a biotinylated rabbit anti-sheep IgG and developed with avidin-conjugated horseradish peroxidase and O-phenylenediamine color indicator. Alternatively, the antigen was sandwiched between monoclonal antibodies designated HPC1 and HPC3 and developed using horse-radish peroxidase-conjugated goat anti-murine IgG. For quantitation of HPC in the culture medium, the polyclonal assay was used because of differences in immunoreactivity of the variants with the two monoclonal antibodies.

SDS-PAGE and Western Blot Analysis—The recombinant glycosylation derivatives were subjected to SDS-polyacrylamide gel electrophoresis (26) on 10% SDS-polyacrylamide gels (acylamide/bis 300:8) under either reducing or nonreducing conditions. Protein was electrophoresed by Coomassie Brilliant Blue staining, or by staining with a murine monoclonal antibody against the major late promoter, a synthetic sequence comprising the spliced leader sequence and the entire protein C insert was isolated. Reconstitution of the complete protein C coding sequence was accomplished via a three-part ligation involving the 3788-base-pair EcoRI/Sall fragment from pLPC, a 730-base-pair Sall/Scl fragment from pLPC, MP18-Q097,
25 °C. One unit of aPC was defined as the amount of aPC required to release 1 μmol of p-nitroanilide in 1 min at 25 °C, pH 7.4, using an extinction coefficient for p-nitroanilide at 405 nm of 9620 M⁻¹ cm⁻¹ (28). Plasma-derived aPC was purified, as previously described (13), and used as a standard for both functional assays.

Purification and Calcium Dependency of HPC—To obtain material for purification and the determination of calcium-dependent elution from anion exchange (15), the recombinant cell lines were grown in a modified mixture of Dulbecco’s modified Eagle’s and Ham’s F-12 media (1.3) containing 1 μg/ml human insulin, 1 μg/ml human transferrin, and 10 μg/ml vitamin K1. Conditioned media were collected, adjusted to a final concentration of 5 mM benzamidine and 4 mM EDTA, pH 7.4, and then assayed on an anion-exchange column (Pharmacia Fast Flow Q resin) as described by Yan et al. (15). After washing with 3 column volumes of 20 mM Tris, 0.15 M NaCl, 5 mM benzamidine, 2 mM EDTA, pH 7.4, and 3 column volumes of 20 mM Tris, 0.15 M NaCl, 5 mM benzamidine, the bound HPC was eluted with 20 mM Tris, 0.15 M NaCl, 10 mM CaCl₂, 5 mM benzamidine, pH 7.4. Residual material was eluted in a high salt buffer of 20 mM Tris, 0.5 M NaCl, and 5 mM benzamidine. The partially purified protein was desalted in a CentriCell unit with a 30,000 M₉ retention filter (Polyscience, Inc. #Y16674-4) following dilution with a buffer containing 20 mM Tris, 0.15 M NaCl, and 0.02% NaN₃, according to the recommended procedure of the manufacturer. The concentration of protein was determined by the BCA assay method (Pierce Chemical Co.) using FQO-purified wt HPC as a standard. Quantification of the wt HPC reference material using 1% BSA as a standard correponding to 1.5 μg of protein/mL gave an absorbance of 0.22 at 280 nm with less than 5% error. Protein purity was determined by densitometer scanning of the obtained UV absorbance at 280 nm with less than 5% error. Enzyme activity was determined by densitometer scanning of the obtained UV absorbance at 280 nm with less than 5% error.

Activation Rates and Kinetics—Activation rates were determined using bovine thrombin (0.20 nm) alone or in complex with a 10-fold molar excess of rabbit thrombomodulin. The activation reaction mix contained 20 mM Tris, 0.15 M NaCl, 0.1 mg/ml BSA, 0.02% NaN₃, 5 mM CaCl₂, 75-kDa soluble recombinant human thrombomodulin TMD1-75 (29). Fully activated HPC was obtained either by incubating the reaction mix overnight with additional TMT or by activating material with 0.05 μM thrombin in complex with soluble recombinant human thrombomodulin TMD1-75 (29). Activation rates were determined by removing aliquots from the reaction mix at various time points, to a 96-well plate containing hirudin at a concentration in 50-fold molar excess of thrombin. The chromogenic substrate, S-2366, was added to each well at a final concentration of 0.75 mM. The rate of amidolytic activity was measured as the change in absorbance units/min at 405 nm in a ThermoMax kinetic microtiter plate scanner (Molecular Devices). Rates were determined by converting change in OD/min to μg of aPC generated by using the specific activities determined for each of the glycosylation mutants and plotting versus activation time.

To determine the Kₐ and kₐ values for wt and Q313 HPC at 3 mM CaCl₂, concentrations from 1 to 20 μM of each were activated with 0.2 nm thrombin/20 nm thrombomodulin, and aliquots were taken over time to determine rates of activation by measuring amidolytic activity as described above. Kₐ and Vₐmax were determined by plotting the slopes of activated PC over time versus the concentration of substrate. Nonlinear regression analysis was performed with EnzFitter software (Elsevier Biosoft, United Kingdom).

For α-1-antitrypsin (α₁-AT) inhibition studies, 100 μg of each purified HPC glycosylation derivative was mixed with 0.167 μM of aPC (which migrates as a single band) and aliquoted into triplicate samples, each of which contained 8 nM α₁-AT in 16 mM HPC, 5 mM CaCl₂, 0.33 μM BSA, 20 mM Tris, pH 7.4, for 45 min. Fully activated HPC at a concentration of 20 nm was incubated with 0 to 71 μM α₁-AT in 3 mM CaCl₂, 150 mM NaCl, 20 mM Tris, pH 7.4, and 1 mg/ml BSA. At selected times, aliquots were removed and the activated protein C activity was determined by amidolytic activity using S-2366 at a final concentration of 1 mM. As previously described (30), apparent first-order rate constants, kₐ, were calculated from the slopes of plots of activated protein C over time in the presence of molar excess of inhibitor. Inhibitor constants, kᵢ, were calculated as kᵢ = kₐ/k[I] (inhibitor) and half-life of protein C was calculated at tᵢ/₄ = 0.69/k[I]. The inhibition of each HPC derivative in plasma was determined by incubating normal human plasma (citrated) with 10 nM activated wt HPC or each HPC derivative. The plasma concentration was 90% (v/v) in the final reaction, with the remaining volume consisting of buffer containing 3 mM CaCl₂, 150 mM NaCl, 20 mM Tris, pH 7.4, and 1 mg/ml BSA. At selected times, aliquots were removed and activated protein C activity was determined by amidolytic activity using S-2366 at a final concentration of 1 mM. The tᵢ/₄ in plasma was determined from decay curves generated using the EnzFitter Software.

RESULTS

Expression and Secretion of Glycosylation Derivatives—Shown schematically in Fig. 1 are the four potential N-linked glycosylation sites in HPC at amino acid positions 97, 248, and 313 and at the unusual consensus site Asn-X-Cys at 329. Each of these sites was singly eliminated by substitution of Gln (Q) for Asn (N) using site-directed mutagenesis (Fig. 1B). Vectors for the expression of wt HPC and each derivative were constructed and introduced into the adenovirus-transformed cell lines 293 (human kidney) and AV12-664 (Syrian hamster tumor). Previously, we have shown that at low levels of expression, both of these cell lines will secrete fully processed rHPC (13, 31) and that stable recombinant 293 cell clones will perform each of the complex post-translational modifications of the protein even at high expression levels (15, 22). To determine if the alterations in glycosylation pattern affected the secretion of HPC, we initially performed transient expression experiments in the two cell lines as described under "Methods." As shown in Fig. 2, the levels of rHPCs Q248, Q313, and Q329 secreted from both cell lines were essentially the same as those observed with the wt HPC. However, the level of rHPC Q097, containing the deletion of glycosylation site 97 in the light chain, was significantly reduced in both cell lines. These data indicate that glycosylation of HPC in the EGF domain is important for efficient secretion of the protein.

For further characterization, stable recombinant 293 cell lines secreting wt HPC and each of the glycosylation derivatives were created by the isolation of hygromycin-resistant clones as previously described (13). For further study, recombinant clones secreting from 5 to 10 μg/ml/24 h in serum-free medium were chosen. To analyze the secreted product, samples of serum-free conditioned medium from representative clones were subjected to SDS-PAGE, and the rHPCs were detected by Western blotting. As shown in Fig. 3, elimination of the glycosylation site in the light chain (Q097) resulted in a reduction in the apparent M₉ of the light chain, and elimination of the glycosylation sites in the heavy chain (Q248, Q313, Q329) resulted in alterations in the distribution of the α, β, and γ subforms. From an examination of the mobility of the heavy chain subforms from each derivative, it was apparent that the M₉ of the carbohydrate side chain at each site must be different (Asn-329 ~ 4000, Asn-248 ~ 3000, Asn-313 ~ 2000). Elimination of glycosylation at Q248 resulted in a 3-4-fold increase in the amount of single chain rHPC secreted, indicating that glycosylation at this site is important for the intracellular removal of the KR dipeptide. Removal of the glycosylation site at 329, with the unusual consensus sequence Asn-X-Cys, resulted in the loss of α-form heavy chain. These data suggest that partial glycosylation at 97 contains an important determinant of the α-subform of HPC secreted by 293 cells.

To obtain a more accurate determination of the relative
The proposed position for the disulfide bonds, the relative locations of the functional domains of the protein, modified amino acids His, and Cys), of the KR dipeptide and thrombin-cleaved activation peptide domain are indicated. The proteolytic cleavage sites for the removal of the signal sequence and propeptide. The glycosylation are shown for each of the four glycosylation sites. The proteolytic cleavage sites for the removal of the heavy chain glycoforms in the derivative 8.75% polyacrylamide gels. The separated proteins were transferred to nitrocellulose, and the HPC was detected using a sheep polyclonal (American Diagnostica) and the Vectastain avidin-biotin complex detection method (Vector Laboratories). STD, secreted wt HPC standard; SC, single chain secreted form; HC, heavy chain; LC, light chain.

FIG. 2. Expression and secretion of glycosylation derivatives of HPC from engineered mammalian cell lines. Comparison of the level of wt HPC and each derivative secreted from human 293 and hamster AV12-664 cells 48 h following transfection with an expression vector containing the indicated form of HPC. The values were made relative to wt HPC for the two cell lines. The HPC expression plasmids were each cotransfected with a vector for the expression of chloramphenicol acetyltransferase. The levels of chloramphenicol acetyltransferase enzyme activity were determined and used to correct for differences in transfection efficiency. The results are the average of three separate experiments.

FIG. 3. Western blot analysis of wt HPC and glycosylation derivatives. The HPC in conditioned medium was subjected to electrophoresis on 8.75% polyacrylamide gels. The separated proteins were transferred to nitrocellulose, and the HPC was detected using a sheep polyclonal (American Diagnostica) and the Vectastain avidin-biotin complex detection method (Vector Laboratories). STD, secreted wt HPC standard; SC, single chain secreted form; HC, heavy chain; LC, light chain.

Table 1 Distribution of heavy chain glycoforms in wt and derivative HPCs

| HPC    | α (%) | β (%) | γ (%) | % of total heavy chain |
|--------|-------|-------|-------|------------------------|
| wt HPC | 54 ± 5| 40 ± 4| 6 ± 3 | 0                      |
| Q097   | 71 ± 4| 27 ± 3| 2 ± 3 | 0                      |
| Q248   | 0     | 68 ± 2| 32 ± 2| 0                      |
| Q313   | 0     | 54 ± 4| 39 ± 4| 7 ± 1                  |
| Q329   | 0     | 84 ± 3| 16 ± 3| 0                      |

Observe non-glycosylated material, suggesting that both of these sites are partially glycosylated. Interestingly, the distribution of the α, β, and γ subforms in Q097 was not identical to those in wt HPC. Apparently, alterations in the EGF glycosylation affect the conformation of the heavy chain and the accessibility of glycosylation site 329 during secretion.

Calcium Dependency and Functional Activities of HPC Glycosylation Derivatives—Using the procedure of Yan et al. (15), the calcium dependency profiles of wt HPC and each of the glycosylation derivatives were compared. In this procedure, fully γ-carboxylated HPC (containing 9 residues of Gla) elutes from an anion-exchange resin in 10 mM CaCl2 (CaCl2 frac-

FIG. 1. Schematic representation of protein C single chain precursor (A) and location of site-specific amino acid changes (B). In panel A, numbers refer to amino acid positions in the mature protein (following removal of the signal sequence and propeptide). The proposed position for the disulfide bonds, the relative locations of the functional domains of the protein, modified amino acids His, and Cys), of the KR dipeptide and thrombin-cleaved activation peptide domain are indicated. The proteolytic cleavage sites for the removal of the KR dipeptide and thrombin-cleaved activation peptide (AP) are shown (see text for details). In panel B, the amino acid substitutions of Gln (Q) for Asn (N) in the consensus sequences for N-linked glycosylation are shown for each of the four glycosylation sites. The KR dipeptide is indicated by the vertical line between the light and heavy chains, and the activation peptide is indicated by the open box on the N-terminus of the heavy chain.

The proportion of the heavy chain glycoforms in the derivative HPCs, purified material (described below) was separated by SDS-PAGE and stained with Coomassie, and the resulting bands were quantitated by densitometric scanning as described under “Experimental Procedures.” The data, summarized in Table I, show that the percentages of wt HPC α, β, and γ subforms were identical to Q313 modified β, γ, and nonglycosylated subforms. These data suggest that the site at 313 is always glycosylated and that the α, β, and γ subforms represent tri-, di-, and monoglycosylated heavy chain, respectively. Upon elimination of either Q329 or Q248, we did not observe nonglycosylated material, suggesting that both of these sites are partially glycosylated. Interestingly, the distribution of the α, β, and γ subforms in Q097 was not identical to those in wt HPC. Apparently, alterations in the EGF glycosylation affect the conformation of the heavy chain and the accessibility of glycosylation site 329 during secretion.
...bound, but can be subsequently eluted with 0.5 M NaCl (NaCl fraction). Very poorly carboxylated molecules do not bind to the column (15, 18). Serum-free conditioned culture medium from each of the recombinant 293 lines was adsorbed to the anion exchange resin, washed, and sequentially eluted as described under "Methods." As shown in Fig. 4A, the elution profile for each of the derivatives was essentially the same as obtained with wt HPC; from 85 to 95% of the total material eluted in the 9-Gla calcium fraction. Thus, alternation in anion exchange resin, washed and sequentially eluted as de-

The activation peptide of each derivative could be removed as indicated by the increase in the functional activities of the wt and derivative rHPCs were prepared with glycosylation derivatives were significantly increased compared with wt aPC, with each derivative having an anticoagulant activity of 2–3 times that of human plasma-derived aPC. We did not attempt to determine kinetic parameters in the crude clotting assay. Alternatively, we determined the kinetic parameters for wt and each of the glycosylation derivatives using the synthetic tripeptide substrate (Table II). While the increases in activity of Q313 and Q248 were primarily due to increased \( k_{cat} \), Q329 displayed a slightly higher affinity (decrease in \( K_a \)). The overall enzyme efficiency \( (k_{cat}/K_m) \) of each heavy chain derivative was approximately twice that of wt HPC.

**Inhibition of the Activated Glycosylation Derivatives—\( \alpha_\text{a}-\text{AT} \) is a major physiologic inhibitor of aPC at the active site. To determine if the glycosylation derivatives had an altered interaction with \( \alpha_\text{a}-\text{AT} \), varying concentrations of purified \( \alpha_\text{a}-\text{AT} \) were incubated with each HPC and the inhibitory activity was determined. The rate of inhibition of wt aPC and each of the derivatives was identical at all concentrations of \( \alpha_\text{a}-\text{AT} \) tested; the pseudo-first-order plots for inhibition of wt aPC and Q313 are shown in Fig. 5. The association rate constants \( (k_b) \) and half-lives were determined for wt aPC and each derivative as described under "Methods" and are summarized in Table IV. The data clearly demonstrated that there were no differences in the kinetics of inhibition by \( \alpha_\text{a}-\text{AT} \). The association rate constants of \( \sim 10 \text{ M}^{-1} \text{s}^{-1} \) and \( T_\text{on} \) values of

**TABLE II**

**Functional amidolytic and anticoagulant activities of HPC glycosylation site derivatives**

Functional activities were determined as described under "Experimental Procedures" using the material purified from the ion-exchange calcium fraction. The first number in parentheses is the fold increase in activity over plasma-derived HPC. The number of independent samples (n) determined in duplicate or triplicate is indicated.

| HPC          | Amidolytic (relative to wt HPC) units/mg | Anticoagulant (relative to plasma wt HPC) |
|--------------|------------------------------------------|------------------------------------------|
| Plasma-derived | ND                                       | 250                                      |
| wt aPC       | 35 ± 5 (1) (n = 7)                      | 325 ± 65 (1.3) (n = 5)                   |
| Q097         | 32 ± 4 (0.9) (n = 10)                    | 303 ± 33 (1.2) (n = 3)                   |
| Q248         | 63 ± 12 (1.8) (n = 8)                    | 669 ± 172 (2.7) (n = 3)                  |
| Q313         | 52 ± 7 (1.5) (n = 9)                     | 627 ± 99 (2.5) (n = 3)                   |
| Q329         | 47 ± 6 (1.4) (n = 9)                     | 516 ± 29 (2.1) (n = 3)                   |

**TABLE III**

**Kinetic parameters for wt and derivative HPCs using synthetic tripeptide Glu-Pro-Arg-p-nitroanilide (S-2366) substrate**

Results are the mean of two experiments with 20 data points used for nonlinear regression analysis. The error values were determined from the best fit curve using the EnzFitter program.

| Enzyme | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) |
|--------|----------|-------------|-----------------|
| wt aPC | 0.03 ± 0.02 | 1.5 ± 0.09 | 4               |
| Q097   | 0.33 ± 0.03 | 1.4 ± 0.06 | 4               |
| Q248   | 0.35 ± 0.03 | 2.9 ± 0.08 | 8               |
| Q313   | 0.33 ± 0.4  | 2.3 ± 0.25 | 7               |
| Q329   | 0.21 ± 0.03 | 1.75 ± 0.08 | 8               |

**FIG. 5. Inhibition of activated wt PC and activated glyco-
sylation derivative Q313 by \( \alpha_\text{a}-\text{AT} \). Samples of pseudoaffinity-purified wt HPC (closed circles, dashed lines) and Q313 (open circles, solid line) were activated with TM/T as indicated under "Methods" and incubated with the indicated amounts of human \( \alpha_\text{a}-\text{AT} \) for various times. Residual activity was determined using synthetic substrate S-2366 (Kabi).**

\( K_a \) of each heavy chain derivative was approximately twice that of wt HPC.

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**FIG. 4. Calcium pseudoaffinity separation of rHPCs on anion exchange chromatography (A) and activation by TM/T complex (B).** In panel A, the wt HPC and glycosylation derivatives in serum-free conditioned cell culture medium were adsorbed to Pharmacia Fast Flow Q resin and sequentially eluted with CaCl\(_2\) and NaCl as indicated under "Methods." Total recoveries of starting material ranged from 87 to 94%. The amount of rHPC in each fraction was determined by enzyme-linked immunosorbent assay. In panel B, samples eluted in the calcium fraction were treated with TM/T for 30 min and compared with untreated samples by SDS-PAGE (8.75% gel, Coomassie-stained). The locations of the wt \( \alpha, \beta, \gamma \) subforms of the heavy chain (HC) and the light chain (LC) are indicated.
27-29 min were essentially identical to the values of 11 M⁻¹ s⁻¹ and 26 min, respectively, obtained for wt aPC by Heeb et al. (30). The half-lives for wt aPC and each derivative in freshly prepared human plasma also were found to be identical (Table IV). Therefore, even though the functional activities of the heavy chain glycosylation derivatives were increased, none were inhibited any more rapidly either in plasma or by active-site inhibition with α₁-AT.

**Activation by Thrombin**—The efficient activation of HPC to aPC by the thrombomodulin-thrombin complex requires a calcium-dependent conformational change in the activation region (33-36). To determine if the removal of glycosylation affected this calcium-dependent activation, we examined the activation rates for wt HPC and each glycosylation derivative using bovine thrombin in complex with either detergent-solubilized rabbit thrombomodulin or recombinant soluble human thrombomodulin TMD1-105 kDa (29). The rates of activation by thrombin were identical with either source of thrombomodulin. As shown in Fig. 6 A, the rates of activation of wt HPC, Q097, Q248, and Q329 were identical. However, the rate of Q313 activation by the TM/T complex was approximately 3-fold higher. We determined the rate of activation for Q313 by the TM/T complex at various calcium ion concentrations (Fig. 6B). There was no significant difference in calcium dependency; activation was inhibited at low concentrations of calcium, and both wt aPC and Q313 had half-maximal stimulatory calcium concentrations of ~0.35 mM.

To determine whether the increase in rate of activation of Q313 by the TM/T complex was due to an increase in affinity for the enzyme-cofactor complex or an increase in turnover, kinetic analyses were performed at 3 mM calcium. As shown in Table V, the $k_{\text{cat}}$ for Q313 was slightly less (~20%) than that of the wt HPC, however, the $k_{\text{cat}}$ for Q313 was substantially reduced. The $k_{\text{cat}}$ obtained for wt HPC is in good agreement to the value of 5 μM previously reported for plasma-derived and recombinant human HPC (35, 36). The overall efficiency ($k_{\text{cat}}/K_m$) with Q313 as substrate was 2.5-fold greater than that observed with wt HPC. We also compared the rates of activation of Q313 and wt HPC by thrombin alone. Because the activation of HPC by thrombin alone is inhibited at physiological levels of calcium, we compared rates both at 3 mM calcium and in the presence of EDTA. Under both conditions, the rate of activation of Q313 was 2.5-3-fold higher than the wt HPC, similar to that observed above for the TM/T complex. Due to the very high $K_m$ for activation of HPC by thrombin alone (33), we did not attempt to establish whether these increased rates were due to $K_m$ or $k_{\text{cat}}$ differences. However, our overall data suggest that the removal of glycosylation at Q313 increases the affinity for thrombin in a calcium-independent manner.

### Table IV

**Kinetic parameters for the inhibition of activated protein C derivatives in normal human plasma and by α₁-antitrypsin**

| aPC   | $k_{\text{cat}}$ (M⁻¹ s⁻¹) (n = 3) | $K_m$ (μM) | $V_{\text{max}}$ (μM) |
|-------|----------------------------------|-----------|-------------------|
| wt aPC| 10.0 ± 1.0                       | 22        | 17.3 ± 0.3        |
| Q097  | 10.2 ± 0.6                       | 24        | 18.4 ± 0.3        |
| Q248  | 10.5 ± 0.6                       | 27        | 17.6 ± 0.5        |
| Q313  | 10.6 ± 0.6                       | 27        | 16.6 ± 0.5        |
| Q329  | 10.0 ± 1.5                       | 22        | 17.6 ± 0.4        |

### Table V

**Kinetic parameters of thrombin-thrombomodulin catalyzed activation of wt HPC and glycosylation derivative Q313**

| Substrate     | $K_m$ (μM) | $k_{\text{cat}}$ (min⁻¹) | $k_{\text{cat}}/K_m$ (µM⁻¹ min⁻¹) |
|---------------|------------|-------------------------|----------------------------------|
| wt HPC        | 6.1 ± 0.45 (n = 3) | 480 ± 14 | 79                                      |
| Q313          | 1.9 ± 0.50 (n = 4) | 390 ± 14 | 200                                     |

### Discussion

In recent years, carbohydrate side chains have begun to receive attention as being integral to the functional properties of glycoproteins. It has become clear that N-linked side chains can control a wide variety of functions, including plasma clearance, signal transduction, receptor activation, intracellular folding, and activity (reviewed in Refs. 37-42). In this paper, we demonstrate that glycosylation at each of the sites on HPC has an effect(s) on the properties of the protein, including dramatic increases in functional anticoagulant activity.

A striking effect of HPC glycosylation appears to be to attenuate the functional activity of the protein; elimination of each glycosylation site in the heavy chain resulted in an increase in both functional amidolytic and anticoagulant activity (Table II). Previous studies (15, 43) have demonstrated that the degree of glycosylation of HPC may affect functional activity, as removal of sialic acid residues by neuraminidase treatment results in a 2–3-fold increase in anticoagulant activity. Sialic acid residues themselves can play a role in the function of a protein as patients with oversialylated fibrinogen...
have extended thrombin times and abnormal fibrin monomer aggregation (44), and desialylation of factor IX results in a loss of coagulant activity (45). It is not clear whether or not the increase in activity of the heavy chain glycosylation derivatives is entirely due to removal of the charged sialic acid residues upon removal of the entire side chain, or if it is simply due to removing steric hindrance of the active site to protein substrates or cofactors. With the synthetic substrate, the increase in activity following removal of glycosylation at 248 and 313 was primarily due to an increase in enzyme kcat. This would suggest that the conformation of the active site is slightly altered in these variants. Of interest, the increased activity of 329 was at least in part due to a decreased Km for substrate. Although the kinetic parameters with synthetic substrate may not reflect the reason for the increased anti-coagulant activities, the data nevertheless suggest that the naturally occurring β-form of HPC is more active. The data further suggest that changes in the ratios of the α- and β-glycans in certain patients (see Ref. 46) may have clinical significance.

In the circulation, aPC is inhibited primarily by two proteins, α1-AT and protein C inhibitor, both of which form a complex via acylation of the active site. As shown in Table IV, there were no differences in the rates of inhibition by α1-AT among any of the HPCs. Additionally, the half-lives of each HPC in plasma were the same, indicating that the interaction with protein C inhibitor was also unaltered by eliminating glycosylation sites. As indicated above, the increase in activity of Q329 appeared in part to be due to an increased affinity. If this increase in active-site affinity was due to removal of steric hindrance, one might have speculated a corresponding increase in the rate of inhibition would have occurred. Most likely, inhibitor docking is the rate-limiting step controlling the rate of inhibition, not active-site availability. Furthermore, our data indicate that the carbohydrate side chains in HPC are not affecting the interaction with either inhibitor in the wt protein.

We previously demonstrated that treatment of an HPC-secreting 293 cell line with tunicamycin, which inhibits N-linked glycosylation, resulted in a 5-10-fold drop in secretion. We now demonstrate that glycosylation at position 97 in the EGF domain of the light chain appears to be the site critically required for the efficient secretion of the protein, as its removal results in a 70–75% drop in secretion (Fig. 2). The effect of N-linked glycosylation on the folding and transport of proteins in the endoplasmic reticulum has been recently reviewed (40), and it is believed that the presence of side chains in strategic positions on the polypeptide are required for correct folding and subsequent efficient transport. Removal of Asn-97 had no effect on the proteolytic processing or γ-carboxylation of the secreted material, however, the ratio of α to β heavy chain increased. It would appear that, in addition to transport, the correct conformation of the EGF domain in the endoplasmic reticulum also affects the conformation of the serine protease domain, thereby altering the accessibility of the glycosylation site at position 329. In support of this, we also have observed an increase in the amount of α-form HPC in two individual mutants of the site of β-hydroxylation in the EGF domain.2 Thus, the EGF domain of HPC appears to play a role in the overall structural properties of the molecule during secretion.

As previously reported, approximately 50% of the nonglycosylated rHPC secreted from tunicamycin-treated cells was single chain (47). We show in Fig. 3 that removal of a glycosylation site at position 248 resulted in a substantial increase in the amount of single chain protein, suggesting that the carbohydrate at this site affects the efficiency of the processing of the KR dipeptide and, like position 97, plays some role in the structural conformation of the molecule during secretion. It is interesting to note that several mammalian cell lines, including CHO, C127, and BHK-21, are not capable of efficient processing of the KR dipeptide (14, 48, 49). Although this cell line dependence for processing may be due to differences in the efficiency of dibasic cleavage, the effect could also be due to the specific carbohydrate side chain processed at Asn-248. As indicated above, the increase in enzymatic activity of Q248 was primarily due to an increase in active site turnover, suggesting that glycosylation at this site also affects the conformation of the fully processed and secreted protein.

Recently, Miletich and Broze (46) demonstrated that an antibody to the region containing Asn-329 did not recognize the α-form but would recognize the β-form, suggesting that the β-form of HPC represents protein not glycosylated at position 329. Our data by site-directed mutagenesis directly confirm this finding. We and others (11, 13, 46) have previously demonstrated that plasma-derived HPC contains from 70 to 80% α-form heavy chain. In contrast, the rHPC secreted from several cell lines typically contains from 50 to 60% α-form. We also have reported that the functional activity of rHPC has a 1.3–1.4-fold higher specific activity when compared with plasma-derived HPC having essentially the same Gla content (13, 15). Possibly, this higher than expected activity can now be explained by the fact that the recombinant material has a higher percentage of high activity β-form protein.

Based on an examination of HPC from warfarin-treated patients, Miletich and Broze (46) proposed that the partial glycosylation at position 329, which has the unusual consensus sequence Asn-X-Cys, is controlled by the rate of translation and thus temporal availability of the site. This hypothesis may not be valid in cell culture with rHPC, as we have not observed a difference in the ratio of α- to β-form HPC in recombinant lines secreting from 50 ng/ml to 30 μg/ml HPC, nor have we observed a difference in the rate of HPC translation upon inhibition of γ-carboxylation using warfarin or Chloro K (18). Alternatively, these authors have suggested that under-carbohydrated molecules are structurally (rather than temporally) more accessible for glycosylation at Asn-329. In support of this, we have found that HPC containing only 7 of the 9 Gla residues (15) possesses an increased proportion of α-chain HPC. Therefore, it would appear that the degree of glycosylation at Asn-329 is influenced by the correct post-translational processing of the Gla domain. Coupled with our observations on the EGF domain, our data indicate that the correct modification of the light chain as a whole influences the conformation of the heavy chain and the accessibility of Asn-329 during secretion into the lumen of the endoplasmic reticulum.

We have demonstrated that the removal of glycosylation at Asn-313 results in an increase in the rate at which thrombin can cleave the activation peptide, primarily due to an increase in affinity of thrombin for this HPC substrate. Lack of glycosylation at Asn-313 did not alter the calcium dependency of activation (Fig. 6). Therefore, the removal of this glycosylation site does not affect the calcium-induced conformational change(s) thought to occur in the activation region. The carbohydrate at 313 likely is near the activation region in the three-dimensional structure and to some extent sterically

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2 B. Gerlitz and B. W. Grinnell, unpublished observation.

3 B. Gerlitz, S. B. Yan, and B. W. Grinnell, unpublished observation.
blocks access to thrombin, whether alone or in complex with its cofactor thrombomodulin. It is also possible that the carbohydrate at 313 binds other domains involved in HPC-thrombin interaction.

Through site-directed mutagenesis, we have begun to understand the role of glycosylation in HPC structure and function. Furthermore, we have demonstrated the potential to engineer a molecule with enhanced catalytic activity through carbohydrate modifications. Our data suggest the possibility that the activity of HPC could be altered in local environments or in disease states due to changes in the degree of glycosylation and/or side chain processing.

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