Registry No. NR, 9013-03-0; FAD, 146-14-5; Mo, 7439-98-7.

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A γ-Carboxyglutamic Acid (γ) Variant (γ^6D, γ^7D) of Human Activated Protein C Displays Greatly Reduced Activity as an Anticoagulant†

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Received May 24, 1990; Revised Manuscript Received August 27, 1990

ABSTRACT: Site-specific mutagenesis has been employed to alter the cDNA of human protein C (PC), such that the γ-carboxyglutamic acid (γ) pair at positions 6 and 7 of the recombinant (r) protein would be changed to aspartic acid residues. This variant, [γ^6D, γ^7D]r-PC, and its wild-type (wt) counterpart have been expressed in human kidney 293 cells. After purification, forms of wtr-PC that were fully γ-carboxylated and β-hydroxylated and of [γ^6D, γ^7D]r-PC that lacked only the two altered γ-residues at amino acid sequence positions 6 and 7 were obtained. Subsequent to its conversion to activated PC (APC), [γ^6D, γ^7D]r-APC displayed a greatly reduced activity in the activated partial thromboplastin time of PC-deficient plasma, as compared to wtr-APC and human plasma APC. In addition, the activity of [γ^6D, γ^7D]r-APC toward inactivation of purified human factor VIII was reduced to less than 5% of that of wtr-APC and human plasma APC. These results, with the first reported mutations at γ-residues of PC produced by recombinant DNA technology, indicate that the paired γ-residues at positions 6 and 7, which are highly conserved in all vitamin K dependent coagulation proteins, are very important to generation of fully functional APC. Additional results demonstrate further that lack of γ-carboxylation at positions 6 and 7 of PC does not substantially affect this same processing reaction at other relevant glutamic acid residues.

Protein C (PC)[1] is a plasma protein that has sequence homology with other vitamin K dependent serine protease zymogens. PC functions as an anticoagulant subsequent to its conversion to activated protein C (APC) by virtue of its limited proteolytic inactivation of cofactors necessary for clot formation, viz., factor V (f-V) and factor Va (f-Va) (Kisiel et al., 1977), as well as factor VIII (f-VIII) and factor VIIIa (f-VIIIa) (Vehar & Davie, 1980), in a reaction that is stimulated by Ca^{2+}, phospholipid (Kisiel et al., 1977), and a cofactor, protein S (Walker, 1980). Maximal activation of PC occurs at the endothelial cell surface as a result of a limited proteolytic event, catalyzed by thrombin, along with the

†Supported by Grant HL-19982 from the National Institutes of Health, a Searle Family Foundation Fellowship (to L.Z.), and the Kleiderer/Pezold Family Endowed Professorship (to F.J.C.).

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[1] Abbreviations: PC, human protein C; APC, activated human protein C; [γ^6D, γ^7D]r-PC(APC), a recombinant protein C (or activated protein C) containing aspartic acid residues substituted for γ-carboxyglutamic acid residues at positions 6 and 7 of the protein C amino acid sequence; γ, γ-carboxyglutamic acid; βOH-D, β-hydroxyaspartic acid; r, recombinant; wt, wild type; DodSO$_4$/PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fmoc, [(9-fluorenylmethyl)oxy]carbonyl.

0006-2960/90/0429-10828$02.50/0

membrane cofactor protein, thrombomodulin, and Ca^{2+} (Esmon et al., 1982).

The entire gene for human PC has been isolated and its nucleotide sequence determined (Foster et al., 1985). Eight exons are present, which code for a leader sequence of 42 amino acids, a light chain containing 155 amino acids, a heavy chain of 262 amino acids, and a dipeptide that connects the two chains. A considerable amount of processing occurs in the generation of the mature protein. This includes cleavage of the leader polypeptide (Foster et al., 1985); glycosylation at four N residues (Kisiel, 1979); γ-carboxylation of nine E residues (Di Scipio & Davie, 1979) and β-hydroxylation of one D residue (Drakenberg et al., 1983) in the light chain of the protein; and cleavage of the dipeptide linker, K^{156}–R^{157}, between the latent heavy and light chains (Beckmann et al., 1985; Foster & Davie, 1984). The intron placements in the gene and the amino acid sequences suggest that the protein is organized into several domains, including the γ-carboxyglutamic acid (γ) region, two consecutive domains homologous to growth factor, an activation peptide, and the catalytic region, the latter of which is homologous to that of other serine proteases (Banyai et al., 1983).

Correctly processed human r-PC (Grinnell et al., 1987; Madden et al., 1990) and r-APC (Erlich et al., 1989) have been expressed from their respective cDNAs in mammalian cell lines and their properties assessed. With the required technology available, we decided to examine the role of particular γ-residues in the function of APC. In this initial report, we present experiments in which one pair of γ-residues, γ^6–γ^7, which is highly conserved in other vitamin K dependent coagulation proteins, has been conservatively altered to D residues, by site-specific mutagenesis, and the importance of these residues to function of the enzyme has been evaluated.

Materials and Methods

Proteins. Purified r-human factor VIII (f-VIII) was obtained from Genentech, Inc. Bovine factor X (f-X) was purified as described previously (Bajaj & Mann, 1973), as was bovine factor IX (f-IX) and factor IXaβ (f-IXaβ) (Amphlett et al., 1979). Human plasma PC was provided by Enzyme Research Laboratories, Inc. (South Bend, IN), and showed 5% impurities when analyzed on $DodSO_4$/PAGE. The *A. contortix* venom activator of PC, Protac, was purchased from American Diagnostica (New York, NY).

Activation of PC was accomplished by incubating 5 μg (4–20 μL, depending upon stock solution concentrations) of the desired PC with 200 μL of Protac (1 unit/mL), 50 μL of 1 M Tris-HCl, pH 7.4, 100 μL of 1 M NaCl, and H_2O to a total volume of 1 mL. The activation reaction time was 1 h at 37 °C, followed by continued incubation at 4 °C for 16 h. The latter step was needed since some inhibition of activation of the PCs was observed with NaCl in the buffer, and we allowed the reaction to proceed until full amidolytic activity (vide infra) of the relevant APC was observed. The above conditions sufficed for full activation of all PCs studied herein.

Murine monoclonal anti-PC, C3, was provided by Dr. John Griffin (La Jolla, CA). Restriction endonucleases were purchased from Fisher Scientific (Springfield, NJ) and BRL (Gaithersberg, MD) and were employed according to the manufacturer's instructions.

Genes for Wild-Type and Mutant PC Molecules. The cDNA coding for wtr-PC was provided by Dr. Earl Davie (Seattle, WA) in pUC119. Between two *Eco*RI sites, the cDNA for PC contained 69 noncoding bases 5′ of the ATG initiation site, 126 bases of leader sequence, 1383 bases coding for the entire PC molecule, and 202 noncoding bases 3′ of the TAG stop codon, this latter region including two poly A signals. We altered the cDNA-containing plasmid by changing the *Xba*I restriction site in the polylinker region of pUC119 to an *Xho*I site, using the synthetic oligonucleotide primer:

5′-GATCCTCTCGAGTCGAC

(the **boldfaced** nucleotide represents the mismatched base). Additionally, an *Nhe*I site was introduced between bases −27 and −26 from the ATG initiation codon of PC, employing the primer

5′-AGTATCTCCACGGCTAGCCCCTGTGCCAG

When the cDNA for PC was excised from pUC119 with *Nhe*I as one of the restriction endonucleases, this latter insertion served the purpose of also removing this cDNA downstream of an additional ATG initiation codon situated at bases −62 to −60 in the 5′ noncoding region, resulting in a cDNA for PC containing 26 bases 5′ from the ATG start sequence.

For insertion into the mammalian cell expression vector, the cDNA was excised from pUC119 by employing an *Nhe*I–*Xho*I restriction digestion and inserted into these same restriction sites of the vector pCIS2M. This latter vector was constructed from the plasmid pCIS (obtained from Dr. Cori Gorman, South San Francisco, CA), which contains, in pML, the human cytomegalovirus (HCMV) major immediate early promoter–enhancer, which is cis-activated by the enhancer (Eaton et al., 1986) and trans-activated (Stinski & Roehr, 1985) by adenovirus (Ad) E1 proteins (Gorman et al., 1989) that are present in the Ad-transformed 293 cells. A splice, followed by a polylinker site, which allows insertion of the gene of interest, is present downstream of the promoter–enhancer region. These areas are followed by a poly A site, an SV40 *ori* (for plasmid replication in cell lines containing T antigen) and promoter, downstream of which is an amplifiable gene (DHFR) for use in systems wherein amplification is desired. We modified the polylinker site of this vector by excision at the *Cla*I–*Xba*I restriction site and inserting the following linker:

5′-CGATTGCTAGCT

TAACGATCGAGATC-5′

This procedure provided a unique *Nhe*I restriction site and restored the *Cla*I and *Xba*I restriction sites, yielding the new vector pCIS2M.

Construction of the variant cDNA coding for [γ^6D, γ^7D]r-PC was accomplished by using the mutagenic synthetic oligonucleotide primer

5′-CTCCTTCCTGGACGACCTCCGTCACAG

Here, mutant colonies were identified by loss of the *Sst*I site at the second base of the codon for L^8. Insertion of this cDNA into pCIS2M was conducted as for the wild-type PC gene.

Transfection in Human Kidney 293 Cells. The wt- and variant r-PC-containing expression vectors (10 μg) were cotransfected with a neomycin gene (1 μg), the latter in a vector under control of the Rous sarcoma virus long terminal repeat promoter, into human kidney 293 cells, grown in DMEM F12 media (Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS). Neomycin selection was used to isolate stable transformants. Single colonies where then picked and grown to confluency, after which the media were tested for the presence of r-PC antigen by Western blotting. r-PC-producing cells were grown in large-scale cell culture in DMEM/F12 media, supplemented with 400 μg/mL G-418 (Gibco)/10% FBS. When confluent, the medium was replaced with DMEM/F12/10% FBS/G-418/vitamin K_1 (5 μg/mL). After 24 h, the cells were washed with a buffer of 4.3 mM

Na_2HPO_4/1.4 mM NaH_2PO_4/2.7 mM KCl/137 mM NaCl, pH 7.3, and the medium was replaced with another containing DMEM/F12/G-418/vitamin K_1 (5 μg/mL). From estimates by Western analyses, the concentration of wtr-PC present was approximately 3 μg/mL of the original medium, and that of [γ^6D, γ^7D]r-PC was approximately 1.5 μg/mL.

Purification of the Recombinant Proteins. The chromatographic method described for purification of wtr-PC by Yan et al. (1990) was employed, with minor operational modifications. Benzamidine hydrochloride (5 mM, final concentration) and EDTA (5 mM, final concentration) were added to the conditioned medium of the 293 cells, after which this solution was passed through a Pharmacia Fast Flow Q (FFQ) anion-exchange column at 4 °C and eluted with a $CaCl_2$ gradient (start solution, 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limit solution, 20 mM Tris-HCl/150 mM NaCl/30 mM $CaCl_2$, pH 7.4), as described (Yan et al., 1990). The $CaCl_2$ was removed by dialysis and Chelex 100 treatment, and the protein was readsorbed to a second FFQ column, after which it was eluted with an NaCl gradient (start solution 20 mM Tris-HCl/150 mM NaCl, pH 7.4; limit solution, 20 mM Tris-HCl/500 mM NaCl, pH 7.4) (Yan et al., 1990). At this point, the wtr-PC had very minor high molecular weight impurities (<2%, estimate) and was used in this condition. The variant protein was purified in this same manner, showing slightly altered column behavior from the wt-protein but resulting in highly homogeneous material.

γ-Carboxyglutamic Acid Determinations. These experiments were performed by amino acid analysis after alkaline hydrolysis. The hydrolyses were conducted with 2.5 M KOH for 20 h at 110 °C and KOH was removed from the samples, as reported earlier (Kuwada & Katayama, 1981). Amino acid analysis, by HPLC, was carried out, after precolumn derivatization with *o*-phthalaldehyde/ethanethiol, employing a 4 mm (i.d.) × 200 mm Nucleosil 5SB column. The exact procedure, including solvent conditions for complete resolution of all acidic amino acids, has been described (Yan et al., 1990). The ratios of γ/D and γ/E, combined with the standard curves, were used to obtain the number of γ-residues per mole of protein. Our exact HPLC components have been published (Chibber et al., 1990).

We employed as reference standards both a commercial *o*-phthalaldehyde-derivatized amino acid standard mixture, as well as a peptide, ANSFLγγLRHSS, representing the first 12 residues of the light chain of PC, synthesized by standard Fmoc chemistry (the N^{α}-Fmoc-γ,γ-ditBu-L-Gla-OH used in the peptide synthesis for placement of Gla in the peptide was chemically synthesized by Dr. Sushil Sharma in this laboratory). Use of this peptide for this purpose allowed us to obtain recovery factors after alkaline hydrolysis of the peptide and to determine very accurate conversion factors of peak areas to concentrations of γ-residues in the hydrolyzed samples. The γ/D ratio in this peptide, of 2.0 (N is converted to D during the hydrolysis), and the concentration response factor of D from commercial standards were employed to obtain the concentration response factor for a γ-residue. Checks of the method were made by performing γ-residue analyses on human plasma PC and bovine plasma factor IX.

β-Hydroxyaspartic Acid Determinations. Standard preparations of *erythro-* and *threo-*βOH-D (*e-*βOH-D and *t-*βOH-D) were generous gifts of Dr. W. T. Jenkins (Bloomington, IN) and Dr. Marvin Miller of this department. For determination of the amounts of *e-*βOH-D and *t-*βOH-D in all PC samples, measured amounts of protein were hydrolyzed with 6 N HCl at 110 °C for 20 h. Acidic amino acids were resolved on anion-exchange HPLC, employing a 4 mm (i.d.) × 200 mm Nucleosil 5SB column, after precolumn modification with *o*-phthalaldehyde/ethanethiol. This procedure has been thoroughly described by Yan et al. (1990). The amounts of *e-*βOH-D and *t-*βOH-D were determined from concentration response curves of the standard samples.

APTT Assays. The activated partial thromboplastin time (APTT) was employed as a whole clotting assay method to test for APC activity, through the creation of functional deficiencies of factor V (f-V) and f-VIII. The APTT assay kit (Sigma Diagnostics, St. Louis, MO) was employed essentially as recommended by the manufacturer. The plasma used was PC-deficient and purchased from the same source. Controls in this assay were performed in the absence of APC and with unactivated PCs, in place of their respective APCs. Test samples consisted of the PC-deficient plasmas, which were incubated with the APCs for 3 min at 37 °C. Clotting times were measured with a commercial fibrometer (Fisher Scientific, Springfield, NJ). Our standard clot times (ca. 45 s) are essentially the same as those of the manufacturer.

Factor VIII Inactivation Assays. For assays of the initial rates of activation of f-X in a complete purified system, and the effect of plasma and r-APCs on these rates, we proceeded as follows. In the first stage of the assay, the abilities of plasma APC, wtr-APC, and the r-APC variant to inactivate the cofactor for f-X activation, f-VIII, were examined. Here, an amount of 10 μL of f-VIII (27 units/mL) was incubated with 8 μL of a sonicated dispersion of 60/40 (w/w) egg phosphatidylcholine/bovine brain phosphatidylserine (PC/PS, 0.75 mM in phosphate), 45 μL of the desired PC or APC (ca. 5 μg/mL, final adjustment to equal amidolytic activities), 25 μL of a 20 mM solution of $CaCl_2$, 7 μL of a 1 M solution of NaCl, and 55 μL of a 25 mM solution of Hepes–NaOH/150 mM NaCl, pH 7.4. The final volume was 150 μL. The times of these incubations were varied, from 0 to 1 h at 37 °C. For activation of the remaining f-VIII, prior to assay in the f-X system, the complete volume of 150 μL of the above f-VIII inactivation mixture was incubated with 10 μL of a solution of 0.1 μM f-IXaβ (for protection of f-VIIIa activity) (Lollar et al., 1984) and 10 μL of a solution of 2 units/mL thrombin (Sigma) at 37 °C for 2 min. The final volume was 170 μL. The amount of f-VIIIa present was then assayed by its ability to stimulate the initial activation rate of f-X. For this stage, an aliquot of 170 μL of the above solution was added to another solution containing, in a spectrometer cuvette at 37 °C, final concentrations of 40 μM (in phosphate) PC/PS, 2.5 mM $CaCl_2$, and 180 μM chromogenic substrate, Bz-L-Ile-L-Glu-Gly-L-Arg-pNA (S2222, Helena Laboratories, Beaumont, TX). The final volume was 790 μL. After recording the baseline for 1 min, substrate hydrolysis was accelerated by addition of 10 μL of a solution of 32 μM f-X. The rate of amidolysis of S2222 by the generated activated f-X (f-Xa) was recorded continually for 2–5 min at 405 nm. The resulting absorbancies were converted to initial activation rates as described previously (Beals et al., 1989).

APC Amidolytic Assays. Amidolytic activities of the APCs were determined by assay against the chromogenic substrate, H-D-Phe-L-Pip-L-Arg-pNA (S2238, Helena Laboratories), at 37 °C. A quantity of 30 μL of substrate (4 mM) was added to a solution containing 80 μL of 1 M NaCl and 40 μL of a buffer consisting of 1 M Tris-HCl, pH 7.4. Next, 20 μL of APC (5 μg/mL) was added and the reaction monitored as above. The total volume was 800 μL.

DNA Analytical Methods. Oligonucleotides were synthesized by using phosphoramidite chemistry on a Biosearch

(San Rafael, CA) Cyclone two-column DNA synthesizer. All reagents were purchased from this same source. The oligonucleotides were purified using the Applied Biosystems (Foster City, CA) oligonucleotide purification cartridges. cDNAs were sequenced by the dideoxy technique (Sanger et al., 1977), using the Sequenase reagent kit (United States Biochemicals, Cleveland, OH). Cell transfections were performed by the calcium phosphate method (Kingston, 1987).

Plasmid DNAs were purified by CsCl/ethidium bromide (EtBr) gradient centrifugation according to Moore (1987), with a Beckman (Palo Alto, CA) L5 65 preparative ultracentrifuge. We used vertical rotor (VTi.65.1) centrifugation for 7 h at 55000 rpm, 15 °C, to separate the DNA bands. After obtaining the desired material from the centrifuge tube, EtBr was removed from the plasmid DNA by extraction into a solution of isopropyl alcohol saturated with CsCl. The DNA was then dialyzed against a buffer of 1 mM Tris-HCl/0.1 mM EDTA, pH 7.1, prior to cell transfections.

The cDNAs and cDNA fragments were purified by excising the appropriate bands after their electrophoretic separation on 1% agarose. Recombinant molecules were created by the method of Struhl (1985).

Single-strand plasmid DNAs were generated as described (Vieira & Messing, 1987) and site-specific mutagenesis was conducted according to Kunkel et al. (1987).

Western Analysis. Protein samples were separated by $DodSO_4$/PAGE (Laemmli, 1970) on 10% (w/v) polyacrylamide gels under nonreducing conditions. The separated protein bands were transferred to Immobilon-P (Millipore, Bedford, MA) according to established procedures (Burnette, 1981) and then incubated at 37 °C for 1 h in 1% (w/v) gelatin (Bio-Rad EIA grade) in TBS (blocking buffer). Our exact conditions for transfer were the following: 4 °C in 25 mM Tris-HCl/200 mM glycine/15% (v/v) methanol, pH 8.3, at 20 V for 12 h. This solution was replaced with another containing 4 μg/mL of monoclonal murine anti-human plasma PC, C3 (Heeb et al., 1988), in blocking buffer, and incubated at room temperature for 2 h with mixing. The filter was washed with three changes of 0.05% (v/v) Tween-20 in TBS at room temperature, over a 15-min period. It was next incubated with rabbit anti-mouse IgG–alkaline phosphatase conjugate (Sigma) in blocking buffer for 2 h at room temperature, with mixing, and then washed as above. Positive bands were visualized after incubations, at room temperature, with the substrate solution (16.5 mg of nitro blue tetrazolium/0.5 mL of 70% (v/v) aqueous DMF/8.5 mg of bromochloroindolyl phosphate in 1 mL of H_2O, which was added to 50 mL of 0.1 M Tris-HCl/0.1 M NaCl/0.005 M $MgCl_2$, pH 9.5).

Results

Human wtr-PC and [γ^6D, γ^7D]r-PC were expressed in human kidney 293 cells with a high-efficiency vector similar to that of Grinnell et al. (1987), who showed that under their conditions full processing of the protein had occurred. Key features of the expression vector that we utilized are illustrated in Figure 1.

The recombinant proteins were purified employing the chromatographic method of Yan et al. (1990). We were able to obtain approximately 1–2 mg of fully processed (in the case of the variant PC, the two mutated Gla residues were lacking) protein per liter of culture medium, after purification. Figure 2 shows nonreduced $DodSO_4$/PAGE electrophoretic analysis of the recombinant proteins, illustrating their high degrees of purity. The commercial plasma-derived PC contained approximately 3–5% impurities. The slightly lower mobilities

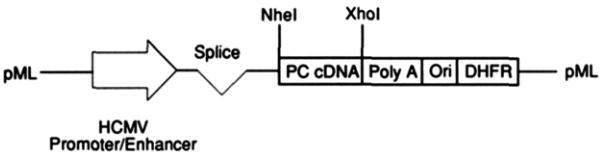

FIGURE 1: Expression vector (pClS2MPC) for wtr-PC and [γ^6D,γ^7D]r-PC. The essential features of this pML-based expression vector are the human cytomegalovirus (HCMV) early immediate promoter–enhancer for initiation of transcription; a chimeric intron, consisting of a 5′ splice sequence from the early immediate region promoter of human cytomegalovirus (Boshart et al., 1985), fused onto a synthetic 3′ splice; the entire coding sequence of wtr-PC or [γ^6D,γ^7D]r-PC, inserted through *Nhe*I–*Xho*I restriction sites; the poly A early polyadenylation sequence; the SV 40 origin of replication (ori); and the cDNA for dihydrofolate reductase (DHFR).

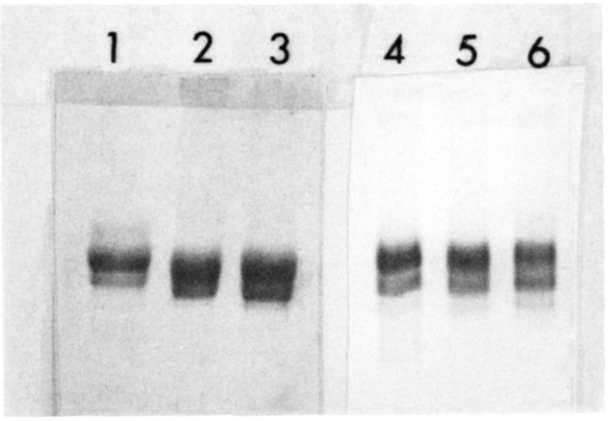

FIGURE 2: Nonreduced electrophoretic analysis of human plasma PC and recombinant PCs. The proteins (20 ng) in gels 1–3 were blotted from a $DodSO_4$/PAGE electrophoretic separation onto Immobilon P and the bands were visualized by immunoassay with the monoclonal antibody C3. The proteins (2 μg) in gels 4–6 were separated by $DodSO_4$/PAGE and stained for protein with Coomassie Blue. Gels 1 and 4, human plasma PC; gels 2 an 5, wild-type r-PC; gels 3 and 6, [γ^6D,γ^7D]r-PC.

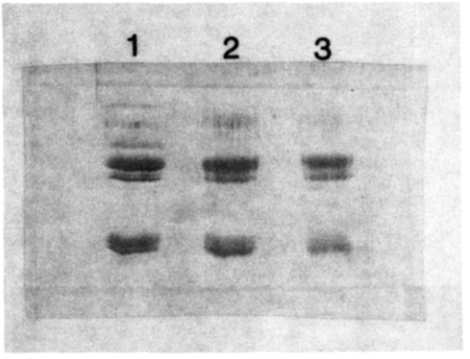

FIGURE 3: Reduced electrophoretic analysis of human plasma PC and recombinant PCs. The reduced proteins (4 μg) were separated by $DodSO_4$/PAGE and stained for protein with Coomassie Blue. Gel 1, human plasma PC; gel 2, wtr-PC; gel 3, [γ^6D,γ^7D]r-PC.

of the 293 cell-derived PCs, as compared to human plasma PC, have been observed previously and are reported to represent differences in glycosylation of these proteins from different cell types (Yan et al., 1990). The results of Figure 2 further demonstrate that both protein bands observed in each of the r-PC samples were immunoreactive toward a monoclonal antibody prepared against human plasma PC. These two bands are normal constituents of purified PC preparations and probably represent glycosylation variants of the proteins. Reduced $DodSO_4$/PAGE gels (Figure 3) show that >90% of each protein is present in the two-chain form, demonstrating that this processing event also has occurred in each of the two recombinant proteins.

Table I: Amino Acid Sequence Analysis of Recombinant Protein C Molecules

cycle	sequence of PC L[a]	sequence of PC H[b]	amino acids identified in plasma PC	amino acids identified in wtr-PC	amino acids identified in [γ^6D, γ^7D]r-PC
1	A	D	A, D	A, D	A, D
2	N	T	N, T	N, T	N, T
3	S	E	S, E	S, E	S, E
4	F	D	F, D	F, D	F, D
5	L	Q	L, Q	L, Q	L, Q
6	γ[c]	E	E	E	D, E
7	γ[c]	D	D	D	D[d]
8	L	Q	L, Q	L, Q	L, Q
9	R	V	R, V	R, V	R, V
10	H	D	H, D	H, D	H, D
11	S	P	S, P	S, P	S, P
12	S	R	S, R	S, R	S, R
13	L	L	L	L	L

[a] Amino acid sequence of the light chain of human plasma PC. [b] Amino acid sequence of the heavy chain of human plasma PC. [c] γ-Carboxyglutamic acid. A trace of E is normally seen when γ-residues are present at the relevant sequence positions. [d] The stoichiometry of D in this cycle was 2-fold greater than that in the same cycles of plasma PC and wtr-PC.

Table II: γ-Carboxyglutamic Acid and β-Hydroxyaspartic Acid Contents of Various Proteins

protein	Gla[a] (mol/mol) expected	Gla[a] (mol/mol) obtained	βOH-D[b] (mol/mol) expected	βOH-D[b] (mol/mol) obtained
PC peptide[c]	2.0	2.0	0	0
bovine f-IX[d]	12.0	12.0 ± 0.3	1.0	1.06 ± 0.05
human PC[e]	9.0	8.7 ± 0.3	1.0	0.92 ± 0.04
wtr-PC[f]	9.0	8.9 ± 0.3	1.0	1.04 ± 0.03
[γ^6D, γ^7D]r-PC[g]	7.0	6.8 ± 0.3	1.0	0.93 ± 0.03

[a] γ-Carboxyglutamic acid. [b] β-Hydroxyaspartic acid. [c] Synthetic peptide consisting of the amino terminal 12-residues of the light chain of human plasma protein C. [d] Bovine plasma factor IX. [e] Human plasma protein C. [f] Wild-type recombinant human protein C. [g] Recombinant human protein C containing D for E substitutions at positions 6 and 7.

Amino-terminal sequencing of each of the PC molecules has been performed. In each case, two residues were liberated in every cycle, in complete agreement with the expected results of sequence analysis of nonreduced two-chain PC. The data are listed in Table I. For wtr-PC, the deduced light-chain sequence is ANSFL$\gamma\gamma$LRHSSL, which is identical with the human plasma PC sequence, proving that the signal sequence has been properly processed in the recombinant protein. For [γ^6D, γ^7D]r-PC, the deduced light-chain sequence was ANSFLDDLRHSSL, proving that the mutations cloned into the cDNA, and verified by nucleotide sequence analysis, were incorporated into the translated protein. The light-chain sequence of [γ^6D, γ^7D]r-PC, through the first eight residues, was confirmed by direct sequence analysis of Immobilon-P blots of $DodSO_4$/PAGE-resolved light chain from reduced protein samples. Additionally, γ-residue analyses of the recombinant proteins, listed in Table II, were in agreement with the expected levels of γ-residues in each of the proteins examined. The βOH-D content of each of the three PC molecules was also measured. The results obtained are also listed in Table II and demonstrate that all of the proteins contained the expected amount of βOH-D. The βOH-D in all three protein samples was present as the erythro form.

Before assay of the anticoagulant properties of human plasma PC, wtr-PC, and [γ^6D, γ^7D]r-PC, each zymogen was converted to its respective APC with Protac, a venom activator that does not require Ca^{2+} (Exner et al., 1985; Orthner et al., 1988) for activation. The resulting enzymes were adjusted

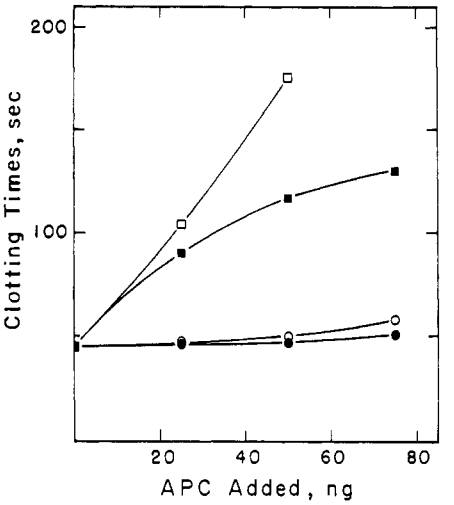

FIGURE 4: Effect of various APCs on the activated partial thromboplastin times of PC-deficient human plasma. The APTT of PC-deficient plasma, in the presence of (□) wtr-APC, (■) human plasma APC, (○) [γ^6D,γ^7D]r-APC, and (●) any of the parent PC molecules used. The clotting times of controls containing buffer, alone, or Protac, alone, were approximately 45 s (ordinate intercept). All PC molecules possessed approximately the same clotting times at each concentration and the average values for the group were plotted (●).

to the same concentrations (and, thus, the same amidolytic activities), prior to comparisons in APTT and factor VIII inactivation assays. It had been shown earlier that full amidolytic activity of r-APC was retained when extensive γ-decarboxylation of this enzyme occurred as a result of expression of r-PC in the presence of warfarin (Grinnell et al., 1987).

As shown in Figure 4, when human plasma APC, wtr-APC, and [γ^6D, γ^7D]r-APC were compared in the APTT coagulant assay, virtually no prolongation of the clotting time of PC-deficient plasma was observed when [γ^6D, γ^7D]r-APC was the source of APC, at a variety of different concentrations. On the other hand, inclusion in the assay of either plasma APC or wtr-APC resulted in the expected increases in the clotting times in the APTT assay. This suggests that [γ^6D, γ^7D]r-APC is essentially inactive toward human plasma f-V and/or f-VIII. Interestingly, wtr-APC is more active than human plasma APC in this assay, a result observed previously (Grinnell et al., 1987). This is not due to differences in the γ-residue content of each protein (Table II) but may suggest a role for oligosaccharide in the activity of APC, since it would be expected that wtr-APC, produced in human kidney cells, would be glycosylated differently than human plasma PC, derived from liver cells.

The conclusion, from the APTT assays, that [γ^6D, γ^7D]r-APC possessed reduced levels of activity toward f-VIII was verified in a direct assay of the cofactor activity of f-VIII in the complete f-X activation system. The strategy of the assay was to preincubate f-VIII and the desired APC for various times, activate the remaining f-VIII with thrombin/Ca^{2+}/PL, with f-IXaβ included to stabilize f-VIIIa, and assay the amount of f-VIIIa present by its cofactor activity in the f-X assay. The results obtained are illustrated in Figure 5 and show that [γ^6D, γ^7D]r-APC, while retaining full amidolytic activity, possessed less than 5% of the activity of the human plasma APC and wtr-APC enzyme toward inactivation of f-VIII.

Discussion

Due to the high level of conservation in all vitamin K dependent coagulation proteins of the amino-terminal proximal paired γ-residues (positions 6 and 7 in human PC), we believed that they would be important in Ca^{2+}-related functions of PC

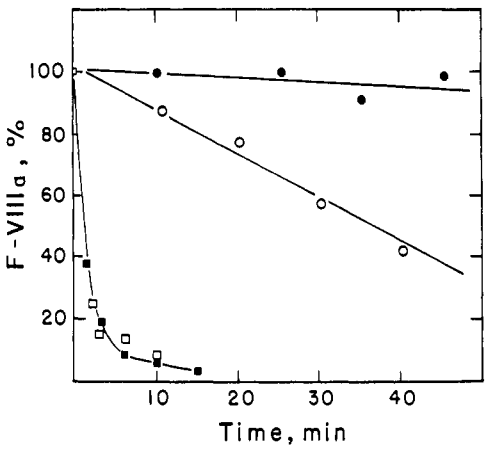

FIGURE 5: Effect of various APCs on the inactivation of human rf-VIII. The desired APC was preincubated with f-VIII, Ca^{2+}, PC/PS, and f-IXaβ for the times indicated on the abscissa. This solution was used to stimulate the activation of f-X, as described under Materials and Methods. The percentage of the original concentration of f-VIIIa present (derived from activation of remaining f-VIII), as measured by its stimulation of f-X activation, is plotted on the ordinate. (●) Control, the amount of f-VIIIa present in the absence of added APC; (■) human plasma APC preincubated with f-VIII; (□) wtr-APC preincubated with f-VIII; (○) [γ^6D,γ^7D]r-APC preincubated with f-VIII.

and APC. We have tested this hypothesis for APC by conservatively mutating the precursor pair of E residues to D, in order that the variant PC would not undergo substantial γ-carboxylation at these positions after processing, and examining the resulting APC in assays that depend on the Ca^{2+} binding integrity of APC. Most interestingly, our data show that lack of γ-carboxylation at positions 6 and 7 does not greatly influence γ-carboxylation at other positions of PC, since the data of Table II clearly demonstrate that all other Gla residues are present in the processed protein and since approximately 80% of the [γ^6D, γ^7D]r-PC adhered to the FFQ column and was eluted with Ca^{2+} in the pool containing seven γ-residues per mole of protein. It has been previously shown that the propeptide region of human PC plays a major role in governing γ-carboxylation of the protein (Foster et al., 1987), but whether other recognition sites for the vitamin K dependent carboxylase exist in the mature protein is a subject of some controversy. The work of Huber et al. (1990) suggests that only residues in the propeptide region define the carboxylateion recognition site, whereas the studies of Price et al. (1987) have led to the conclusion that this same recognition site also consists of amino acids in the Gla domain, particularly those involving residues 16–22 of the prothrombin sequence. While the current investigation does not resolve this controversy, it does demonstrate that γ-carboxylation of residues 6 and 7 in the PC sequence is not requisite for γ-carboxylation of succeeding E residues.

The wt and variant recombinant proteins have been highly purified and rigorously characterized to confirm that the changes in cDNA in the variant were incorporated into the protein and that each protein was otherwise correctly processed, in that the KR dipeptide at positions 156 and 157 of single-chain PC was removed in at least 90% of the molecules, providing the two-chain form of the PC; the signal polypeptide and propeptide were correctly removed; γ-carboxylation at nonmutated sites in the amino terminus of the light chain was essentially complete; and a single residue of βOH-D was present in each of the proteins.

Virtually no prolongation of the APTT time of PC-deficient plasma occurred when [γ^6D, γ^7D]r-APC was added to PC-deficient plasma, whereas substantial increases in the clotting time of this same plasma resulted from addition of either human plasma APC or wtr-APC (Figure 4). This indicates that the variant r-APC did not effectively inactivate f-VIII (or f-VIIIa) and/or f-V (or f-Va), reactions that require functional Ca^{2+} binding, and/or did not interact productively with the cofactor for these inactivations, protein S, which would also lead to longer APTT times. A previous study (Ohlin et al., 1988) has apparently shown that another Ca^{2+}-binding region, which is influenced by βOH-D at position 71 of PC, also is important to the maintenance of a normal APTT time of plasma, via its interaction with another cofactor, protein S. However, these latter studies are difficult to interpret since they are based upon investigations with wtr-APC and [D^{71}E]r-APC molecules with otherwise incomplete γ-carboxylation (4.3 mol/mol and 6.7 mol/mol, respectively). In our laboratory, as well as in the work of Yan et al. (1990) with incompletely γ-carboxylated recombinant PCs from a variety of cell lines and in the study of Esmon et al. (1983) with a human plasma PC derivative in which the γ-domain was proteolytically removed, it has been found that incomplete or totally deficient γ-carboxylation of wtr-APC or plasma APC leads to a large loss of its activity in the APTT times of human plasma. Thus, whether βOH-D is important to maintenance of the APTT times of plasma relies upon further investigation with appropriate APC molecules.

In order to examine directly the activity of [γ^6D, γ^7D]r-APC toward f-VIII and the possible importance of this reaction in the defect in its anticoagulant activity, we performed direct assays of the ability of this variant to catalyze inactivation of f-VIII in a purified system. The results of Figure 4 clearly show that [γ^6D, γ^7D]r-APC is much less effective than human plasma APC and wtr-APC in this regard, displaying an activity of less than 5% of either the wtr-APC or human plasma APC. This confirms that the large loss of prolongation of the APTT time of PC-deficient plasma by [γ^6D, γ^7D]r-PC is due to its decreased activity toward f-VIII, most likely a result of compromised Ca^{2+} binding properties of the variant r-APC. It is highly likely that [γ^6D, γ^7D]r-APC would possess similarly diminished activity toward inactivation of f-V, a cofactor that functions as does f-VIII in the analogous prothrombin activation reaction. However, the very low level of activity of [γ^6D, γ^7D]r-APC toward f-VIII is sufficient in itself to account for the results in the overall APTT assay, despite the fact that at least one additional mechanistic consideration is likely important. Interestingly, the higher activity of wtr-APC, as compared to that of human plasma APC, observed in the APTT assay, was not observed to the same extent in the purified factor X activation assay. This suggests that the additional events that occur in the APTT assay are more affected by wtr-APC than by its plasma counterpart.

It is clear that a large portion of the Ca^{2+} bound to PC and APC requires the presence of γ-residues (Amphlett et al., 1981) and that Ca^{2+} binding is required for inactivation of f-VIIIa by APC. The studies presented herein suggest that the γ-pair, at positions 6 and 7 of APC, which is highly conserved in other vitamin K dependent coagulation proteins, would be among those residues required for full expression of the anticoagulant activity of APC. With the very conservative γ-deleted variant prepared here, it is not possible to assess whether either, or both, of these γ-residues is completely and directly required for the anticoagulant activity of the enzyme, since it is possible that the proximal carboxyl groups of the substituted D residues may provide a means for some Ca^{2+} binding to occur through a chelation-type mechanism, similar

to a single γ-residue, and/or that a low level of δ-carboxylation could take place on the D residues (Hubbard et al., 1989) that have been substituted for the E residues. Either of these possibilities may allow for the low anticoagulant activity of [γ^6D,γ^7D]r-APC observed. Continuing longer term investigations in this laboratory are aimed at the resolution of issues such as this, as well as at assessment of other amino acid residues that are important in the anticoagulant activity of APC. The studies reported here with the first recombinant γ-residue deletions in PC provide much encouragement that these goals will be accomplished.

ACKNOWLEDGMENTS

We acknowledge with gratitude the assistance provided by Dr. Cori Gorman (Genentech) in establishing the 293 cell expression system in our laboratory, Dr. Betty Yan (Lilly Research Laboratories) for providing an advance copy of her publication on the chromatographic purification of wtr-PC, and Dr. Bakshy Chibber, Director of the Biosciences BioCore Facility of the University of Notre Dame, for assistance with some of the analytical protein techniques.

Registry No. Blood coagulation factor XIV, 60202-16-6; γ-carboxyglutamic acid, 53445-96-8; activated blood coagulation factor XIV, 42617-41-4; blood coagulation factor VIII, 9001-27-8.

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