Comparison of amino acid sequence of bovine coagulation Factor IX (Christmas Factor) with that of other vitamin K-dependent plasma proteins

(blood coagulation/zymogens/γ-carboxyglutamic acid/vitamin K/homology with serine proteases)

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ABSTRACT   The amino acid sequence of bovine blood coagulation Factor IX (Christmas Factor) is presented and compared with the sequences of other vitamin K-dependent plasma proteins and pancreatic trypsinogen. The 416-residue sequence of Factor IX was determined largely by automated Edman degradation of two large segments, containing 181 and 235 residues, isolated after activating Factor IX with a protease from Russell's viper venom. Subfragments of the two segments were produced by enzymatic digestion and by chemical cleavage of methionyl, tryptophyl, and asparaginyl-glycyl bonds. Comparison of the amino acid sequences of Factor IX, Factor X, and Protein C demonstrates that they are homologous throughout. Their homology with prothrombin, however, is restricted to the amino-terminal region, which is rich in γ-carboxyglutamic acid, and the carboxyl-terminal region, which represents the catalytic domain of these proteins and corresponds to that of pancreatic serine proteases.

Factor IX (Christmas Factor) is the zymogen of a serine protease that participates in the middle phase of the intrinsic pathway of blood coagulation (1). Like several other plasma proteins— i.e., prothrombin, Factor X, Factor VII, Protein C, and Protein S—Factor IX requires vitamin K for its biosynthesis (2–4). Individuals lacking Factor IX (Christmas disease or hemophilia B) show bleeding symptoms essentially identical to those of classic hemophilia or hemophilia A (Factor VIII deficiency) (5, 6). The activity of Factor IX is also depressed in the plasma of patients treated with coumarin analogs, but in such cases the effect can be reversed by the administration of vitamin K (6).

Bovine and human Factors IX have been purified and characterized (7–12). The bovine molecule, a glycoprotein with a molecular weight of 55,400, is composed of a single polypeptide chain (7). Human Factor IX is also a glycoprotein, with a molecular weight of 57,000 and an amino-terminal sequence nearly identical to that of the bovine molecule (11). Like other vitamin K-dependent plasma proteins, bovine and human Factors IX contain in the amino-terminal region approximately 12 γ-carboxyglutamic acid residues (4).

In the presence of calcium ions, Factor XI_a converts Factor IX to Factor $IX_{a\beta}$ by the cleavage of two internal peptide bonds, Arg-Ala and Arg-Val, releasing an activation glycopeptide of 10,000 daltons (13, 14). Factor IX can also be converted to Factor $IX_{a\alpha}$ by a protease from Russell's viper venom (RVV-X), but in this case only the internal Arg-Val bond is cleaved and no activation peptide is released (14, 15). Both Factor $IX_{a\alpha}$ and Factor $IX_{a\beta}$ are composed of two chains held together by at least one disulfide bond. Either form of activated Factor IX converts Factor X to active Factor X_a in the presence of activated Factor VIII, calcium ions, and phospholipid (15).

Previous comparison of the partial sequence of bovine Factor IX with the sequences of bovine prothrombin and Factor X revealed homologous regions, indicating that these three proteins may have evolved from a common ancestral protein (16, 17). Recently, the light and heavy chains of bovine Protein C, another vitamin K-dependent plasma protein, have been found to be homologous with Factor X (18, 19). In the present communication, the complete sequence of Factor IX is presented. Comparison with the sequences of Factor X, Protein C, and prothrombin reveals areas of homology as well as regions lacking obvious similarity. The experimental details of the sequence determination will be published elsewhere.

METHODS

Factor IX, isolated from bovine plasma (7) and converted to Factor $IX_{a\alpha}$ by a protease from Russell's viper venom (15), was obtained through the courtesy of K. Fujikawa. Factor $IX_{a\alpha}$ was reduced with dithioerythritol and S-alkylated with 4-vinylpyridine. The two peptide chains (segment N and segment C) were separated on a column of SP-Sephadex C-25 by applying a linear gradient of sodium formate buffers in the presence of 7 M urea (20).

Segment N (residues 1–181) was divided into two fragments (CB I and CB II), by cleavage with cyanogen bromide under conditions that avoid cleavage at γ-carboxyglutamyl residues (21). Tryptic digestion of fragment CB I (residues 1–61) yielded three peptides composed of residues 1–11, 12–43, and 44–61 (see Fig. 1). Fragment CB II (residues 62–181) was cleaved with hydroxylamine, 2-(2-nitrophenyl)-3-methyl-3-bromoindole (BNPS-skatole), or trypsin before and after succinylation. Chymotryptic digestion of fragment CB II yielded arginine-containing peptides, which were located by reaction with phenanthrenequinone.

Segment C (residues 182–416) was similarly cleaved into three fragments with cyanogen bromide. After amino groups had been blocked by succinylation, fragment CB III (residues 182–349) was digested with trypsin. The isolated tryptic peptides were aligned in the sequence by their overlap with arginine-containing chymotryptic peptides. Fragment CB IV (residues 350–392) was further digested with trypsin or chymotrypsin. Fragment CB V (residues 391–416) was digested with chymotrypsin.

Large fragments were separated on Sephadex columns by using as solvents 9% (vol/vol) formic acid or 0.1 M ammonium bicarbonate, pH 8. Small peptides were separated on columns of Dowex 50, Dowex 1, or DEAE-Sephadex, followed by high-voltage paper electrophoresis.

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           5          10         15         20         25         30
   1   Y N S G K L γ γ F V R G N L Y R γ C K γ γ K C S F γ γ A R γ

  31   V F γ N T γ K T T γ F W K Q Y V D G D Q C E S N P C L N G G

  61   M C K T·D I N S Y E C W C Q A G F E G T N C E L D A T C S I

  91   K N G R C K Q F C K R D T D N K V V C S C T D G Y R L A E D

 121   Q K S C E P A V P F P C G R V S V S H I S K K L T R↓A E T I

 151   F S N T N Y E Ⓝ S S E A E I I W D Ⓝ V T Q S Ⓝ Q S F D E F S

 181   R↓V V G G E D A E R G Q F P W Q V L L H G E I A A F C G G S

 211   I V N E K W V V T A A H C I K P G V K I T V V A G E H N T E

 241   K P E P T E Q K R N V I R A I P Y H S Y Ⓝ A S I N K Y S H D

 271   I A L L E L D E P L E L N S Y V T F I C I A D R D Y T N I F

 301   S K F G Y G Y V S G W G K V F N R Ġ R S A S I L Q Y L K V P

 331   L V D R A T C L R S T K F S I Y S H M F C A G Y H E G G K D

 361   S C Q G D S G G P H V T E V E G T S F L T G I I S W G E E C

 391   A M K G K Y G I Y T K V S R Y V N W I K E K T K L T
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Composition

21	Ala	A		12	Gln	Q		19	Leu	L		33	Ser	S
18	Arg	R		28	Glu	E		30	Lys	K		26	Thr	T
23	Asn	N		34	Gly	G		3	Met	M		8	Trp	W
18	Asp	D		9	His	H		18	Phe	F		18	Tyr	Y
22	Cys	C		23	Ile	I		13	Pro	P		28	Val	V
12	Gla	γ												

M_r without carbohydrate = 47,279   Number of residues = 416

M_r with carbohydrate = 55,400
[by sedimentation equilibrium (7)]

FIG. 1.   The amino acid sequence of bovine coagulation Factor IX. Circled symbols for asparagine (N) identify four sites of carbohydrate attachment; the symbol γ denotes γ-carboxyglutamic acid (Gla). The vertical arrows separating residues 146–147 and 181–182 indicate sites of cleavage by Factor XI$_a$; only the latter site is readily cleaved by the protease from Russell's viper venom (RVV-X). The amino acid composition beneath the sequence defines the one-letter code notations.

Amino acid analyses were performed with a Durrum amino acid analyzer (model D-500). Tryptophan was determined after alkaline hydrolysis (22). Automated sequence analyses were performed with a Beckman sequencer (model 890 B) by the method of Edman and Begg (23) as modified by Hermodson et al. (24, 25), or with a Sequemat by the method of Laursen (26). Peptides were attached to the solid phase via the carboxyl-terminal homoserine or lysine or the α-carboxyl group. Small peptides were analyzed in the sequencer in the presence of Polybrene (27). Phenylthiohydantoin derivatives of the amino acids were identified by gas/liquid chromatography (24) or by high-performance liquid chromatography (28). Carbohydrate attachment sites were identified both by solid phase Edman degradation and by isolation of small glycopeptides from subtilisin digests.

RESULTS AND DISCUSSION

The complete amino acid sequence of bovine Factor IX is shown in Fig. 1. The protein is composed of a single polypeptide chain of 416 amino acid residues with carbohydrate linked to four asparaginyl residues (i.e., residues 158, 168, 173, and 261). The amino acid composition, as calculated from the sequence in Fig. 1, corresponds to a molecular weight of 47,279 without carbohydrate. Measurements of sedimentation equilibrium indicate a molecular weight of 55,400 (7); the difference between these two values provides an estimate of the carbohydrate content.

Twelve residues of γ-carboxyglutamic acid (Gla) were found within the amino-terminal region of the molecule at positions 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36, and 40. Similar findings have been reported for three other vitamin K-dependent pro-

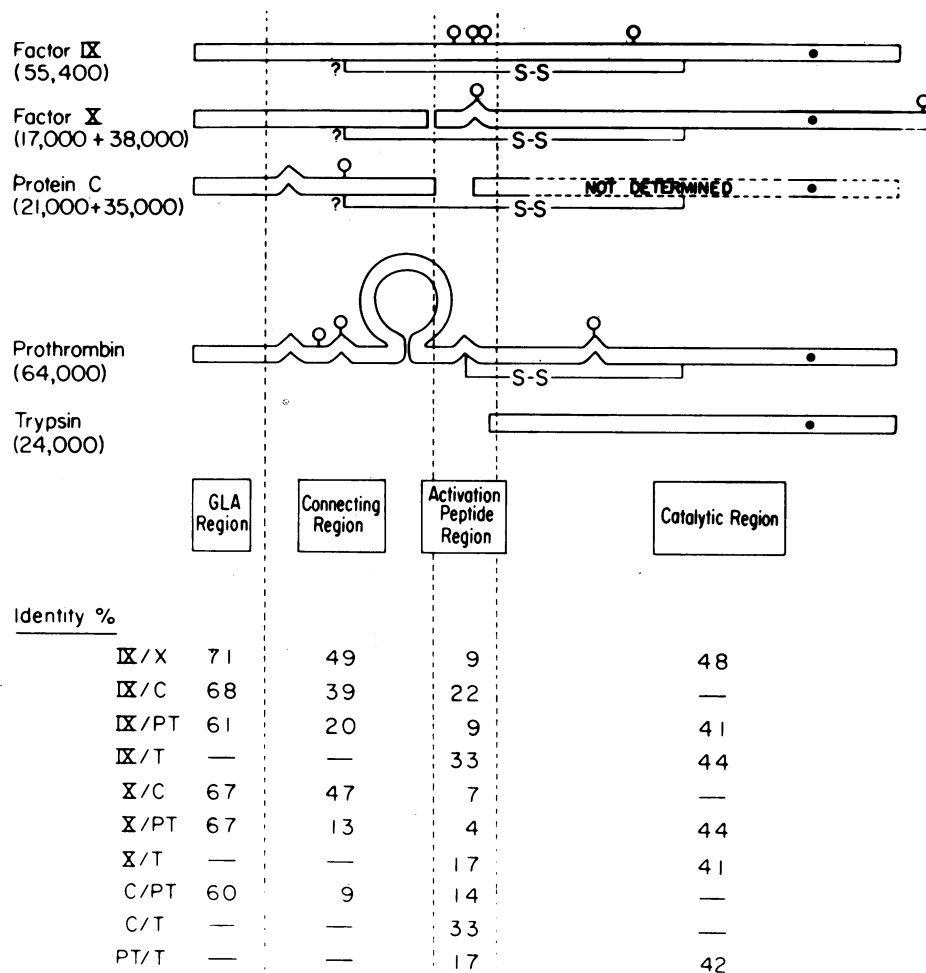|	GLA Region	Connecting Region	Activation Peptide Region	Catalytic Region
IX/X	71	49	9	48
IX/C	68	39	22	—
IX/PT	61	20	9	41
IX/T	—	—	33	44
X/C	67	47	7	—
X/PT	67	13	4	44
X/T	—	—	17	41
C/PT	60	9	14	—
C/T	—	—	33	—
PT/T	—	—	17	42

FIG. 2. Schematic comparison of the zymogens of five bovine serine proteases. The lengths of the bars are proportional to the lengths of the polypeptide chains. Wrinkles or loops are included to indicate the adjustments that are necessary to place in register the homologous amino- and carboxyl-terminal regions (Fig. 3). The circles above each bar indicate the sites of carbohydrate attachment. The portions of each chain homologous with the carboxyl-terminal 235 residues of Factor IX are aligned at both the sites of activation (residues 181–182 in Factor IX) and the serine residue at the active site (●; residue 366 in Factor IX, Fig. 1). Factor X and Protein C each contain two polypeptide chains connected by a disulfide bond; the other three proteins are each single chains in their zymogen forms. The precise positions of the interchain disulfide bonds in the upper three proteins have not yet been established. The complete sequence of both chains of Protein C have been determined (18, 19), but only one was available when this figure was prepared.

For comparison with Fig. 3 and the text, four regions of these proteins are delineated by the vertical broken lines. The percentages of identical residues in these regions are summarized at the bottom, where each line represents comparison of one pair of regions (PT and T denote prothrombin and trypsinogen, respectively).

teins (18, 29, 30). Residues 7 and 8 of Factor IX had previously been identified as γ-carboxyglutamyl residues by Bucher *et al.* (31). Initially γ-carboxyglutamyl residues were tentatively identified (32) by characterizing sites of anomalous cleavage with cyanogen bromide (21) and by high-performance liquid chromatography of their phenylthiohydantoins, which are very poorly extracted from the sequencer cup by chlorobutane. More recently it has been found that methylamidation or ethylamidation of Gla-containing peptides before sequence analysis facilitates extraction and unambiguous identification of their phenylthiohydantoins[‡].

Factor XI$_a$ activates Factor IX by cleaving two internal peptide bonds, Arg-Ala and Arg-Val (13). These peptide bonds are now identified as joining residues 146–147 and 181–182, as shown by the arrows in Fig. 1. Because the protease from Russell's viper venom also activates Factor IX by cleaving only the Arg-Val bond, this cleavage must be sufficient to generate

an active conformation of Factor IX$_a$. The acidic character of the activation peptide (residues 147–181, which include five glutamyl, two aspartyl, and several sialic acid residues, but only one arginyl residue) would account for the differences in electrophoretic migration between Factors IX and IX$_a$ (13). There is some chemical similarity between the tripeptide sequences, Leu-Thr-Arg (residues 144–146) and Phe-Ser-Arg (residues 179–181), preceding the two sites of cleavage by Factor XI$_a$. In this respect the activation is analogous to that of bovine prothrombin by Factor X$_a$ in which the two sites of cleavage are preceded by two identical tetrapeptide sequences (33). In the activation of Factor IX, Factor X, and Protein C by the protease from Russell's viper venom, the sequences *following* the sites of cleavage are also similar—i.e., Val-Val-Gly-Gly (15), Ile-Val-Gly-Gly (34), and Ile-Val-Asp-Gly (35), respectively.

In Fig. 2, the amino acid sequence of bovine Factor IX is compared diagrammatically with the sequences of other bovine vitamin K-dependent proteins and with bovine pancreatic trypsinogen. Among these proteins, Factor IX, prothrombin, and trypsinogen are each composed of a single chain, whereas

‡ W. Baschong, K. Titani, H. Neurath, and L. H. Ericsson (1979) *Abstracts of the Heidelberg Conference on Methodological Progress in Protein Sequence Analysis.*

GLA REGION

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              1                              20                                40
IX   [1-44]   Y N S G K L γ γ F V R - G N L γ R γ C K γ γ K C S F γ γ A R γ V F γ N T γ K T T γ F W K Q
X    [1-43]   A N S - F L γ γ - V K Q G N L γ R γ C L γ γ A C S L γ γ A R γ V F γ D A γ Q T D γ F W S K
C    [1-43]   A N S - F L γ γ - L R P G N V γ R γ C S γ γ V C γ F γ γ A R γ I F Q N T γ D T M A F W S K
PT   [1-44]   A N K G F L γ γ - V R K G N L γ R γ C L γ γ P C S R γ γ A F γ A L γ S L S A T D A F W A K
VII  [1-13]   A N - G F L γ γ L L - P G S L
S    [1-13]   A N T - L L γ γ - T K K G N L
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CONNECTING REGION

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              45                                      60                                      80
IX   [45-81]  Y V D G D Q C E S - - - - - - - - - N P C L N G G M C K T D I N S Y E C W C Q A G F E G T N
X    [44-80]  Y K D G D Q C E G - - - - - - - - - H P C L N Q G H C K N G I G D Y T C T C A E G F E G K N
C    [44-88]  Y S D G D Q C E D R P S G S P C D L P C C G R G K C I H G L G G F R C D C A E G W E G R F
PT   [45-86]  Y T A - - - C E S A R N P R E K L N E C L E G N C A E G V G M N Y R G Ⓝ V S V T R S G I E
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              82                                      100                            115
IX   [82-115] C E L D A T - - - - - - - - - - - C S I K N G R C K Q F C K R D T D N K V V C S C T D G Y
X    [81-115] C E F S T R E I - - - - - - - - - C S L D N G G C D Q F C - R E E R S E V R C S C A H G Y
C    [89-124] C L H E V R F S - - - - - - - - Ⓝ C S A E B G G C A H Y C - M E E E G R R H C S C A P G Y
PT   [87-131] C Q L W R S R Y P H K P E I Ⓝ S T T H P G A D L R E N F C R N P D G S I T G P W C Y T T S
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              116                   130                   146
IX   [116-146] R L A E D Q K S C E P A V P F P C G R V S V S H I S K K L T R
X    [116-140] V L G D D S K S C V S T E R F P C G K F T Q G R S
C    [125-155] R L E D D H Q L C V S K V T F P C G R L G K R M E K K R K T L        (112 residues)
PT   [132-274] P T L R R E E C S V P V C G Q D R V T V E V I P R S G G ——————————————————— E G R
                                                                      |◄——————————————————————►|
                                                                              S-FRAGMENT
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ACTIVATION PEPTIDE REGION

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              147                              160                            175
IX   [147-175]                     A E T I F S N T N Y E Ⓝ S S E A E I I W D Ⓝ V T Q S Ⓝ Q S
X    [H 1-45 ] W A I H T S E D A L D A S E L E H Y D P A D L S P T E S S L D L L G L Ⓝ R T E P S A G E D
C    [H 1-8  ]                                                                         D T N Q V D Q K
PT   [275-317]         T S E D H F Q P F F N E K T F G A G E A D C G L R P L F E K K Q V Q D E T Q K E L F E
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              176       181
IX   [176-181] F D E F S R
X    [H46-51 ] G S Q V V R
C    [H 9-14 ] D Q L D F R
PT   [318-323] S Y I E G R
T    [ 1-6  ]  V D D D D K
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FIG. 3. A possible alignment of regions (defined in the text, and in Fig. 2) of Factors IX, X, and VII with those of Protein C, Protein S, prothrombin (PT), and trypsinogen (T). Gaps are included to optimize homology. The percentage of identity in these regions is summarized in Fig. 2. The sequences of Factor X (36, 37), trypsinogen (38), Protein C (18, 35), prothrombin (33), Factor VII, and Protein S (39) were reported previously. Residue numbers for Factor IX are placed above the sequence; residue numbers are also given to the left of each horizontal line (the symbol H denotes the heavy chain of Factor X or Protein C). Circled residues denote sites of carbohydrate attachment.

Factor X and Protein C, as isolated from plasma, have two-chain structures. All these proteins, except trypsinogen, are glycoproteins. The known sites of carbohydrate attachment are indicated in Fig. 2. A single disulfide bond provides an inter-chain bridge in each of the activated plasma proteins.

The comparison of these proteins in Fig. 2 and Fig. 3 is facilitated by arbitrarily dividing the molecules into four regions: a "Gla region," a "connecting region," an "activation peptide region," and a "catalytic region." The Gla region consists of the amino-terminal 44 residues of the single chains of Factor IX and prothrombin and of the light chains of Factor X and Protein C. There are ten or twelve γ-carboxyglutamyl residues present in this region, but no glutamyl residues, apparently due to hepatic cotranslational or posttranslational modification of glutamyl residues (2). Present evidence indicates that Factor IX, Factor X, and prothrombin all bind Ca^{2+} ions via their γ-carboxyglutamyl residues, and this links each protein to phospholipid derived from platelets during fibrin formation *in vivo*. As shown in Fig. 3, the sequences of these regions of the four proteins are homologous, corresponding to 60–71% identity.

The segment following the Gla region and preceding the activation peptide region is denoted as a connecting region. The various vitamin K-dependent proteins contain a relatively large number of disulfide bonds in this portion of the polypeptide

chains. The corresponding segment of prothrombin is composed of two tandem "kringle structures" (33) that are homologous, as indicated both by their sequences and by the location of their disulfide bonds. It seems unlikely that these portions of Factor IX, Factor X, and Protein C form kringle structures because they differ from prothrombin both in chain length and in cystine content. When the four proteins are aligned as shown in Fig. 3, there is greater similarity among Factor IX, Factor X, and Protein C (39–49%) than between any of these three proteins and prothrombin (9–20%). Thus it is probable that in these regions the conformations of Factor IX, Factor X, and Protein C will bear greater resemblance to each other than to the conformation of prothrombin.

In the region of the activation peptide, the polypeptide chains of the four vitamin K-dependent proteins differ from each other in both size and sequence. During activation, segments are excised from Factor IX, Factor X, and Protein C, whereas in prothrombin the activation peptide remains attached to the catalytic region through a disulfide bridge. There is some indication of common ancestry in the identical tetrapeptides Thr-Ser-Glu-Asp in prothrombin (residues 275–278) and in the heavy chain of Factor X_1 (residues H5–H8), but no such similarity is evident in Factor IX and Protein C (see Fig. 3). A common general feature of the activation peptides is their

marked hydrophilicity and acidity, particularly in trypsinogen. There is no consistent pattern of carbohydrate attachment in these proteins.

In the catalytic regions, the four vitamin K-dependent proteins are clearly homologous with each other and with pancreatic serine proteases. All of these proteins contain the residues identified with the catalytic function of the pancreatic serine proteases (40). Among the four vitamin K-dependent proteins, 41–48% of the residues occur in corresponding loci of their catalytic domains (Fig. 2).

According to the alignment of the four plasma proteins shown in Fig. 2, homology is evident in the amino-terminal 40 residues and in the carboxyl-terminal 250 residues, whereas the intervening regions ($\approx$125 residues in Factor IX) of the four proteins bear less resemblance to each other. This is most clearly apparent by comparing the single chains of Factor IX and prothrombin, which differ in length, disulfide content, sites of carbohydrate attachment, and the sequences of their activation peptides and connecting regions. Similar comparison with Factor X and Protein C is more difficult because these proteins contain two chains. When the shorter chains are aligned with each other and with Factor IX and prothrombin, by placing their Gla regions in register, the connecting regions show little similarity with prothrombin. Similarly, when the longer chains of Factor X and Protein C are aligned with each other and with Factor IX and prothrombin, the catalytic regions are homologous but the activation peptides show little similarity with each other. It is not known whether Factor X and Protein C originally contained homologous segments separating their connecting regions and activation peptide regions. If such segments were present, they have been removed during processing and secretion.

One is left with indications that the terminal regions of these proteins bear traits of common ancestry and that large internal regions of Factor IX, Factor X, and Protein C have diverged from each other rather than from a prothrombin-like prototype. The result is a family of proteins that differ in molecular weights, in the number of polypeptide chains, and in biological specificity but bear clear indications that they have evolved from a common ancestor by a complex evolutionary pathway.

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