Rat Factor X Is Synthesized as a Single Chain Precursor Inducible by Prothrombin Fragments*

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Factor X in plasma is a γ-carboxylated two-chain glycoprotein which, in active form, plays a pivotal role in blood coagulation. We have utilized purified rat Factor X antibody, coupled to Sepharose, to isolate and characterize Factor X in rat liver, plasma, and hepatoma cells. Rat factor X is synthesized as a single chain precursor (Mr = 63,000). It is this form which undergoes vitamin K-dependent carboxylation in rat liver microsomes. Only after secretion is Factor X converted into its two-chain mature form. Single chain X synthesis and secretion in hepatoma cells is enhanced by vitamin K. The NH2-terminal γ-carboxylated prothrombin fragments which induce prothrombin synthesis (Graves, C. B., Munns, T. W., Carlisle, T. L., Grant, G. A., and Strauss, A. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4772-4776) also induce single chain X synthesis by hepatoma cells. We propose that synthesis of all vitamin K-dependent proteins may be regulated by this common control mechanism.

Blood coagulation Factor X is one of 7 vitamin K-dependent γ-carboxylated glycoproteins secreted by the liver (1-3). Factor X is the zymogen of the serine protease Factor Xa and, as isolated from plasma, contains a glycosylated heavy chain (Mr = 38,000-48,000, 307 amino acid residues) and a γ-carboxylated light chain (Mr = 18,000, 140 amino acid residues) bound together through a disulfide link (4-8). The complete primary amino acid (9) and oligosaccharide structures (10) of bovine Factor X have been determined. The light chain, particularly in its NH2-terminal γ-carboxyglutamyl-containing region, possesses extensive homology (60-70%) to the other vitamin K-dependent proteins (prothrombin, Factors VII and IX, and proteins C, S, and Z). The heavy chain of Factor X has significant homology to the catalytic region of these same vitamin K-dependent proteins and to other serine proteases. However, Factor X differs from five of the six other vitamin K-dependent liver proteins, in that it has two peptide

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inhibitors were added (11). After washing, the cells were harvested, extracted with detergent (2% Triton X-100), and an intracellular supernatant (100,000 × g for 1 h) was prepared (11). [3H]Leucine-labeled Factor X was adsorbed to and eluted from Sepharose-conjugated antibodies to rat Factor X according to the procedures described for the isolation of [3H]-labeled rat prothrombin (11). [3H]Factor X was quantitated following electrophoresis in 7.5% SDS-PAGE (19). Further details of these procedures are described in the text and legends of appropriate figures and tables.

**Vitamin K-dependent Carboxylation of Factor X in Vitro** (20)—Male rats (200-500 g) were treated with sodium warfarin (5 mg/kg, intraperitoneal) 18 h prior to sacrifice. Excised liver tissue was homogenized in 3 volumes of 0.25 M sucrose-0.025 M imidazole, pH 7.2, and centrifuged at 10,000 × g for 10 min at 4°C. The resultant postmitochondrial supernatant was processed to yield microsomal fractions following centrifugation at 5,000 × g for 1 h at 4°C. Microsomal pellets were homogenized in 0.25 M sucrose-0.025 M imidazole, pH 7.2, 0.2 M KCl-Triton X-100 (2% v/v). Solubilized microsomal supernatant fractions were recovered after centrifugation at 5,000 × g for 1 h at 4°C. Vitamin K-dependent protein carboxylation was assayed in 1.0 ml of soubilized microsomes (0.8 ml), NADH (1 mg), Na[14C]CO3 (50 μCi), and vitamin K (50 μg). Following incubation for 30 min at 37°C, [3H]-labeled Factor X was isolated, by absorption to anti-Factor X Sepharose, and characterized (SDS-PAGE) as referred to above.

**Assay for Induction of Factor X Synthesis**—The synthesis of Factor X was evaluated following exposure of H-35 cells to bovine prothrombin fragment 1 as described by Graves et al. (12). Confluent cells were maintained under serum-free conditions for 18 h. Vitamin K (0.6 μg/ml) was added for 1.5 h prior to refeeding cells with fresh medium containing vitamin K (0.1 μg/ml) and 0.13 M sucrose-0.025 M imidazole. After a 1-h incubation period, the cells were refed with leucine-free polypeptide-free medium containing [14C]leucine (50 μCi/ml). Following a 1-h labeling period, the quantity of secreted [3H]Factor X was determined after immunochromatography and electrophoresis in SDS-PAGE.

**Assay for Factor X Activity**—Rat plasma was assayed for Factor X activity using Factor VII and X-deficient bovine plasma according to the method described by Bachman et al. (21).

**RESULTS**

**Immunospecific Retention and Identification of Rat Factor X**—Factor X was purified from rat plasma according to the procedures outlined by Novoa et al. (17). Following electrophoresis on SDS-PAGE under nonreducing conditions, the purified protein migrated as a single diffuse band possessing an apparent Mr = 63,000 (Fig. 1). After reduction of the Factor X preparation, stained gels showed two protein bands with apparent Mr = 45,000 and 22,000. These molecular weight estimates are consistent with the values reported for Factor X isolated from human and bovine plasma (22).

To characterize Factor X antigenic material in plasma, we utilized immunochromatography. To compare Factor X antigenic material with other vitamin K-dependent plasma proteins, antisera to total barium-adsorbable plasma proteins was coupled to Sepharose. Analysis of the plasma proteins retained by this immunoadsorbant, by SDS-PAGE, under nonreducing conditions, revealed eight major protein bands (Fig. 2A) ranging in Mr = 20,000-75,000. The most prevalent and largest Mr band (band 1) comigrated with a rat prothrombin standard. Based upon molecular weight, the major doublet (bands 4 and 5) with Mr = 45,000 would be appropriate for Factor X, heavy chain; Factor IX; and Factor VII (1). Similarly, the smallest Mr doublet (bands 7 and 8) would be appropriate for protein C and Factor X light chains. As a control, rat plasma was adsorbed to nonimmune serum coupled to Sepharose. No measurable protein could be eluted from this adsorbant, and analysis of the eluent by SDS-PAGE revealed no stainable protein bands (data not shown).

Factor X antibody was purified from the antisem made against barium-adsorbable proteins by affinity chromatography to the purified antigen (see Fig. 1). The Factor X antibody was then coupled to Sepharose and plasma proteins retained by this immunoadsorbant analyzed to test its specificity (Fig. 2). SDS-PAGE of the proteins eluted from this adsorbant (Fig. 2B) revealed three protein bands possessing Mr = 63,000, 45,000, and 22,000 and corresponding to bands 3, 4, and 7 in Fig. 2A. Proteins in these three bands (compare with Fig. 2A) were quantitatively retained by anti-Factor X Sepharose. The other γ-carboxyl glutamate-containing peptides (bands 1, 2, 5, 6, 8), including prothrombin (band 1), were not adsorbed. As revealed by integration of the peaks in the absorbance tracing of the stained gel, 40% of the protein was the 63,000-dalton peptide; 40%, the 45,000-dalton peptide; and 20%, the 22,000 dalton peptide. The two smaller peptides are identical in size with Factor X subunits purified by conventional procedures from plasma. Since the 63,000 dalton peptide was specifically adsorbed to anti-Factor X Sepharose, this result demonstrated that a single chain form of Factor X is present in plasma.

We also compared the Factor X activity of plasma with that of the plasma proteins which did not bind to Factor X immunoadsorbant. Factor X activity was abolished from the plasma after immunoadsorption (data not shown). We concluded that the Factor X antibody used in subsequent experiments was specific for Factor X antigen.

**Identification and Characterization of Factor X Synthesis in H-35 Cells**—Having demonstrated the efficacy of using anti-rat Factor X-Sepharose to isolate rat plasma Factor X, we studied the synthesis and secretion of Factor X in the H-35 culture system. Samples obtained from vitamin K-supple-
adsorbed to Factor graph and absorbance SDS-PAGE. A photograph of the stained gel is shown above the major stained bands discussed in the text. Peak specifically adsorbed and eluted were analyzed as above. The gel photo-

heating, the immunospecifically retained peptides were analyzed by conjugated antibodies made against total barium-adsorbable plasma sorbants against total barium-adsorbable proteins to those designated in X rat prothrombin standard, while peaks from intracellular preparations by immunoabsorption re-

strongly suggest that, in hepatoma cells, Factor X is synthesized cells labeled with [3H]leucine for 2 h were subjected to immunospecific adsorption. Gel profiles of immunoreactive extracellular Factor X showed a discrete peak of radioactivity corresponding to a $M_r = 63,000$ (Fig. 3A) with very minor peaks of $M_r = 30,000$ and 23,000. Labeled proteins isolated from intracellular preparations by immunoadsorption revealed a single radioactive peak (Fig. 3B) of identical molecular weight (i.e. 63,000). Two-chain Factor X would have been detected if it were present intracellularly in amounts equal to 1% of the total immunoreactive material. These results strongly suggest that, in hepatoma cells, Factor X is synthesized and secreted as a single chain peptide.

As shown above (Fig. 2B), rat plasma immunoreactive Factor X material contains the mature subunits, but in addition, a single chain form totally resistant to reduction is present. These results imply that in vivo single chain X is proteolytically processed to the mature two-chain form after its secretion into the extracellular space.

**Vitamin K-dependent Carboxylation of Rat Liver Microsomal Factor X in vitro**. Representative radioactivity profiles of $^{14}C$-labeled proteins derived from solubilized rat liver microsomes treated with vitamin K and Na$_2$CO$_3$. Details of these procedures are described under “Materials and Methods.” A represents the profile of $^{14}C$-labeled proteins isolated following immunoprecipitation with antisera elicited against barium-adsorbed rat plasma proteins. B represents the profile of $^{14}C$-labeled proteins isolated following immunospecific absorption to anti-rat prothrombin (PT) Sepharose (○○○○) and anti-rat Factor X Sepharose (●●●●). Electrophoretic analysis in SDS-PAGE was performed as described in the legend to Fig. 1. Gel slices were 2 mm, counting efficiency was 85%.
activity with $M_r = 75,000$ and 63,000, respectively. Significantly, these radioactive peaks are clearly separable and show no evidence of immunologic cross-reactivity between prothrombin antibody and Factor X antibody. These findings identify the presence of single chain Factor X in hepatic tissue and further demonstrated that this protein undergoes in vitro post-translational vitamin K-dependent carboxylation.

Single Chain X Undergoes Asparagine-linked Core Glycosylation—Titani et al. (9) determined the sequence of bovine plasma Factor X and identified the presence of asparagine- and threonine-linked carbohydrates at residues 36 and 300 of the heavy chain. Assuming that rat Factor X possesses similar oligosaccharide structures, we examined the ability of tunicamycin, an antibiotic which blocks the formation of asparagine-linked oligosaccharides, to alter the electrophoretic mobility of the one-chain Factor X synthesized by H-35 hepatoma cells. As revealed in Fig. 5, treatment of cells with tunicamycin produced a shift, corresponding to a decrease of 15% in the electrophoretic mobility of the heavy chain. These results demonstrate that the 63,000-dalton peptide contains asparagine-bound oligosaccharide. No lower molecular weight immunoreactive material was observed, suggesting that significant proteolytic degradation of single chain Factor X had not occurred. Moreover, the quantity of extracellular Factor X material was decreased by 15% in the presence of tunicamycin. In agreement with our observations regarding the synthetic regulation of prothrombin, these findings imply that asparagine-linked oligosaccharides enhance the rate of secretion of single chain factor X.

The Effects of Vitamin K on Factor X Synthesis and Secretion in H-35 Cells—Our previous studies indicated that in the presence of vitamin K synthesis of prothrombin and its secretory rate were increased in hepatoma cells (11, 16). Subsequent studies revealed that induction of prothrombin synthesis was not due to vitamin K itself but was mediated by NH$_2$-terminal prothrombin fragments generated following the limited proteolysis of extracellular prothrombin (12). Induction required the $\gamma$-carboxyglutamate residues present in this region of active prothrombin. The results presented in Table I reveal that the synthesis of Factor X is similarly affected. During a 2-h pulse of H-35 cells with $[^{3}H]$leucine, the presence of vitamin K increased the quantity of secreted single chain Factor X by 1.7-fold. Vitamin K also altered the ratio of newly synthesized intracellular and extracellular single chain X. When compared to the K-deficient state, the presence of the vitamin reduced intracellular single chain X by 27%. Thus, as with prothrombin synthesis, vitamin K accelerates the secretion of newly synthesized single chain X.

In view of the ability of serine protease inhibitors to decrease the production of prothrombin in response to vitamin K (12), the synthesis of Factor X was evaluated following exposure of cells to benzamidine-HCl and trasylol. The results of these studies revealed little change in the quantity of Factor X secreted by vitamin K-deficient cells (Table II). On the other hand, incubation of vitamin-supplemented cells with the inhibitors decreased the extracellular accumulation of Factor X by as much as 40%. Under these conditions the quantity of secreted Factor X was nearly identical with the values exhibited by vitamin-depleted-cells. These results indicate that the inhibition of proteolytic enzymes blocks vitamin K-dependent induction of Factor X synthesis.

Induction of Single Chain Factor X Synthesis by Bovine

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Intracellular $[^{3}H]$protein*</th>
<th>Extracellular $[^{3}H]$protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-K</td>
<td>+K</td>
<td>-K</td>
</tr>
<tr>
<td>$[^{3}H]$Factor X</td>
<td>109</td>
<td>80.4 (0.73)$^{cd}$</td>
</tr>
<tr>
<td>$[^{3}H]$Prothrombin</td>
<td>69.3</td>
<td>35.8 (0.52)</td>
</tr>
</tbody>
</table>

* dpm of intracellular $[^{3}H]$Factor X or $[^{3}H]$prothrombin X $\times 10^{-1}$. Total trichloroacetic acid-precipitable intracellular $[^{3}H]$protein was 2.23 $\times 10^{5}$ dpm.

$^a$ +K denotes cells labeled in the presence (+) or absence (−) of 0.5 pg/ml of vitamin K.

$^{cd}$ dpm of extracellular $[^{3}H]$Factor X or $[^{3}H]$prothrombin $\times 10^{-1}$. Total trichloroacetic acid-precipitable extracellular $[^{3}H]$protein was 0.53 $\times 10^{5}$ dpm.

$^d$ Fold increases over −K experiment are indicated in parentheses.

**Table II**

Effect of protease inhibitors on the synthesis and secretion of Factor X in H-35 cells

<table>
<thead>
<tr>
<th>Inhibitor (concentration)</th>
<th>Extracellular $[^{3}H]$Factor X as percentage of extracellular $[^{3}H]$protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.17</td>
</tr>
<tr>
<td>Benzamidine-HCl (50 pg/ml)</td>
<td>0.19 (1.12)$^{b}$</td>
</tr>
<tr>
<td>Trasylol (20 pg/ml)</td>
<td>0.18 (1.06)</td>
</tr>
</tbody>
</table>

$^a$ +K denotes cells labeled in the presence (+) or absence (−) of vitamin K.

$^b$ Fold differences compared to control values are shown in parentheses. These differences are representative of between two and six experiments.
TABLE III
Induction of Factor X synthesis following exposure of H-35 cells to bovine prothrombin fragment 1

<table>
<thead>
<tr>
<th></th>
<th>Extracellular [TH] Factor X (dpm x 10^-5)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine prothrombin Fragment 1</td>
<td>130^a</td>
<td>1.84</td>
</tr>
</tbody>
</table>

^a Total trichloroacetic acid-precipitable extracellular protein was 5.2 x 10^6 dpm.
^b Total trichloroacetic acid-precipitable extracellular protein was 5.4 x 10^6 dpm.

Prothrombin Fragment 1—We previously observed that trasylophan and benzamidine-HCl (12) depress prothrombin production by inhibiting secreted protease activities which degrade extracellular prothrombin. Since the single chain Factor X protein does not undergo extracellular degradative (Fig. 3A), the results presented in Table II suggested that Factor X synthesis might be regulated by peptide fragments derived from some other vitamin K-dependent protein, i.e. prothrombin. In light of (i) the extensive homology in the NH2-terminal portions of prothrombin and Factor X (1, 9) and (ii) the ability of NH2-terminal peptides of prothrombin to induce prothrombin synthesis (12), we evaluated the synthesis of Factor X following exposure of H-35 cells to exogenous bovine prothrombin fragment 1 (residues 1–156). The results (Table III) revealed that the quantity of secreted [3H]labeled Factor X was increased by 1.8-fold. These observations demonstrated that the synthesis of at least these two vitamin K-dependent clotting factors is induced by proteolytic fragments derived from prothrombin.

DISCUSSION

Immunological procedures were developed for the isolation of Factor X present in rat liver, plasma, and cultured H-35 hepatoma cells. Using purified anti-rat Factor X antibodies conjugated to Sepharose, we have demonstrated that (i) Factor X is synthesized and secreted as a single chain protein containing an apparent Mr = 63,000, (ii) proteolytic conversion to the two-chain form occurs in the extracellular space, (iii) the amount of newly synthesized single chain X secreted is one-half that of prothrombin in the presence of adequate vitamin K, and (iv) single chain X synthesis is induced by NH2-terminal y-carboxyglutamate-containing peptides derived from prothrombin.

Since all of these conclusions were based on immunological isolation of Factor X antigen, proof of the specificity of the antibody was essential. Evidence that the antibody was specific for Factor X included the isolation of two-chain Factor X from rat plasma (Fig. 2), removal of Factor X activity from rat plasma, and lack of cross-reactivity with prothrombin and other y-carboxylated proteins (Fig. 4).

A number of investigations have demonstrated that Factor X is recovered from plasma as a two-chain disulfide-bonded protein (4–9). Although single chain forms of human (23) and bovine Factor X (24) have been reported, their existence has not been confirmed (1). The employment of the immunological techniques described in this report allowed the direct and rapid isolation of Factor X, thus reducing the possibility of nonphysiologic proteolytic processing of Factor X. Using this method, both single chain and two-chain Factor X were found in plasma.

Assuming that two-chain Factor X normally circulates in vivo, our results strongly suggest that the single chain molecule is converted to the two-chain form after secretion. This interpretation is based upon these findings: (i) only the single chain and two-chain species were detected in rat plasma; and (ii) Factor X is synthesized and secreted as a single chain molecule in the H-35 culture system (Fig. 3). While intracellular proteolytic processing of proteins is common, extracellular proteolytic processing of single chain proteins to heteropolymers is unusual. Known examples of such processing include rat liver haptoglobin (25); procomplement factors C4, C5, and C6 (26); sucrase-isomaltase (27); and urokinase (28).

The extent of Factor X purification from bovine plasma has led to estimates of 0.2–0.5 µM as its plasma concentration (17, 22). The normal level of bovine prothrombin has been reported as approximately 1.0–2.7 µM (29). The circulating concentration of prothrombin thus exceeds that of Factor X by 2- to 2.5-fold. Our results (Table II) indicate that, in the dependence of vitamin K, the quantity of extracellular Factor X is one-half that of prothrombin. If in liver, as in the hepatoma, synthesis and secretion of single chain X is one-half that of prothrombin, the differences in plasma concentration (2- to 10-fold) may be explained by differences in rates of degragation or by not taking into account the single chain Factor X present in plasma. Estimates of two-chain Factor X and prothrombin half-lives are highly variable (29, 30), i.e. 6–80 h. Given these variable estimates of steady state plasma concentrations and half-lives, the synthetic ratio which we report is plausible, although we had anticipated that Factor X synthesis would be far less than prothrombin synthesis.

One goal of the present studies was to compare regulation of Factor X synthetic rates with regulation of prothrombin synthesis. We have previously shown that rat prothrombin synthesis is induced up to 5-fold (12) by bovine prothrombin fragment 1 (residues 1–156). Under fully induced conditions, prothrombin synthesis was 0.5% of the extracellular protein. However, the base-line or control rate of prothrombin synthesis varied from 0.08–0.24% of the extracellular protein depending upon the amount of endogenously generated prothrombin fragments present in the medium. In order to obtain the full 5-fold induction, the base-line rate of synthesis was suppressed by removing endogenous extracellular prothrombin fragments. This was accomplished through refeeding the cells twice with fresh medium containing protease inhibitors (12). When we tested the effect of bovine prothrombin fragment 1 on single chain Factor X synthesis (Table III) the induction of Factor X synthesis was 1.8-fold. In the same experiment prothrombin synthesis was induced 2-fold, and the base-line rate of synthesis was high (0.24%). Under these experimental conditions, induction of prothrombin and Factor X synthesis was not dramatic, probably because endogenously generated prothrombin fragments were present in the medium. Further manipulations will be necessary to provide definitive evidence that single chain Factor X synthesis is induced by prothrombin fragment 1. However, this result raises the possibility that synthesis of all vitamin K-dependent clotting factors is regulated by a common control mechanism. There are two features in the coagulation process which suggest a pivotal role for prothrombin in the regulation of vitamin K-dependent clotting proenzyme biosynthesis. First, the conversion of prothrombin to thrombin is one step in coagulation which is shared in the extrinsic and intrinsic clotting systems (1, 31). Second, unlike other vitamin K-related proteins, the NH2-terminal y-carboxyglutamic acid containing 1 portion of prothrombin is released upon activation. Because no disulfide
bridges connect this region to thrombin, vitamin K-dependent peptides of prothrombin dissociate from activation complexes. We postulate that these fragments might serve as key regulatory elements which induce the subsequent and rapid restoration of appropriate concentrations of γ-carboxyglutamate-containing proteins in the plasma.

In summary, we have shown that coagulation Factor X is initially made as a single chain form which undergoes γ-carboxylation and core glycosylation intracellularly. Single chain Factor X synthesis in rat hepatoma cells is half that of prothrombin which also induces prothrombin synthesis in these cells.

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