Design of Constructs for the Expression of Biologically Active Recombinant Human Factors X and Xa

KINETIC ANALYSIS OF THE EXPRESSED PROTEINS*

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David L. Wolf†‡, Uma Sinha†, Tom E. Hancock†, Pei-Hua Lin†, Terri L. Messier‡,
Charles T. Esmon‡**, and William R. Church†

From †COR Therapeutics, Inc., South San Francisco, California 94080, ‡Howard Hughes Medical Institute, Departments of Pathology and Biochemistry, University of Oklahoma Health Sciences Center, and the Section of Cardiovascular Biology, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, and the **Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405-0068

Activation of vitamin K-dependent plasma proteases occurs by specific interaction with components of the blood coagulation cascade. In this report, we describe the direct expression and enzymatic characterization of the human coagulation zymogen factor X and its activated form, factor Xa, from transformed Chinese hamster ovary fibroblast cell lines. Expression was achieved using either a full-length factor X cDNA or a unique mutant factor Xa cDNA. The functional factor Xa precursor contained a novel tripeptide bridge in place of the native 52-amino acid activation peptide. This mutation allowed for intracellular processing and secretion of the activated form of factor X. Secreted recombinant factors X (rX) and Xa (rXa) were purified by sequential anion-exchange and immunoaffinity chromatography. The enzymatic activities of factors rX and rXa were compared with those of plasma factors X and Xa in three independent assay systems. In comparison to human plasma factor X, the amidolytic, prothrombinase complex, and plasma clotting activities of factor rX were 50, 85, and 43%, respectively. The corresponding comparative activities for factor rXa were 32, 64, and 48%, respectively. The ability to directly express mutant forms of biologically active human factor X will facilitate the structure/function analysis of this important blood coagulation protein and may lead to the development of novel coagulation inhibitors.

Factor X, a vitamin K-dependent blood coagulation glycoprotein, is the precursor for factor Xa, the enzyme component of the prothrombinase complex (1). In plasma, factor X circulates as a zymogen in the form of a two-chain polypeptide consisting of a 17-kDa light chain joined by a disulfide bridge to a 45-kDa heavy chain. During biosynthesis, a number of post-translational processing events occur (1, 2). These include endopeptidic cleavage, glycosylation, and conversion of select glutamic acid residues in the amino terminus of the light chain to γ-carboxyglutamic acid by a vitamin K-dependent carboxylase. Calcium ion and phospholipid binding by factor Xa is a direct consequence of light chain carboxylation (2).

The extrinsic coagulation pathway, composed of tissue factor and factor VIIa, and the intrinsic pathway, consisting of factors IXa and VIIIa, catalyze the proteolytic activation of factor X to its active species, factor Xa (1). Both pathways require calcium-dependent complex assembly on phospholipid membranes. Factor X is also activated in vitro by a factor X activator isolated from Russell's viper venom (RVV) (3). Proteolytic cleavage at Arg541-Lle543 in a 52-amino acid activation peptide from the amino terminus of the heavy chain lead to the formation of the active enzyme, factor Xa. The catalytic site of factor Xa is located on the heavy chain (3, 4).

Factor Xa forms a macromolecular complex with factor Va on negatively charged phospholipid surfaces. This complex catalyzes the activation of prothrombin to thrombin, an important enzyme that elicits both pro- and anticoagulant responses during cardiovascular trauma (1). Factor Xa also forms a ternary complex with factor VIIa and a lipoprotein-associated coagulation inhibitor (5, 6). Formation of the factor Xa-lipoprotein-associated coagulation inhibitor-factor VIIa complex has been shown to inhibit the extrinsic coagulation pathway.

One approach for the structure/function characterization of topographical sites on factors X and Xa could be accomplished by site-directed mutagenesis. In this report, we describe the direct expression in Chinese hamster ovary (CHO) cells of recombinant human factors X (rX) and Xa (rXa) and compare their biological activities to the plasma factors. This approach demonstrates a strategy for intracellular zymogen activation and establishes the foundation for a more systematic analysis of this important coagulation enzyme.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**—Menadione sodium bisulfite, p-nitrophenyl p′-guanidino benzenezoate HCl, bovine brain phosphatidyl serine, hen egg phosphatidylcholine, rabbit brain cephalin, bovine factor X and VII-X-deficient plasma, and bovine serum albumin were obtained from Sigma. Chromogenic substrates 2,2-azinodi(3-ethylbenzthiazolinesulfonic acid), Chromozym X (N-naphthoxy carbonyl-L-norleucylglycylarginyl-4-nitranilideacetate); M13mp19, and all DNA-modifying enzymes were purchased from Boehringer Mannheim. Human factor

1 The abbreviations used are: RVV, Russell's viper venom; CHO, chinese hamster ovary; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
X-deficient plasma, human antithrombin III, and human prothrombin were obtained from American Bioproducts Co. Human factors X, Xa, Va, and human α-thrombin; and purified RVV factor X activator were purchased from Haemagen Diagnostics Technologies. Human and bovine factor X activators were purified as described (7). Lipofectin reagent and G418-neomycin were from Bethesda Research Laboratories-GIBCO. Coagulation reagents thromboplastin C (composed of rabbit brain-derived thromboplastin) and actin FS (composed of soy phosphatides in eelagic acid) were from Baxter. All reagents were of analytical grade.

Construction of Human Factor X and Xa-expressing Cell Lines—The HindIII-XbaI fragment of plasmid Bluscript containing the full-length human factor X cDNA (8) was subcloned into the XbaI site of m13mp19. Oligonucleotide site-directed mutagenesis was performed as described by Kunkel et al. (9) (utilizing the following oligonucleotides: 5'-ACCTTGGAACGGGAAAAAGATCGTGGGAGGCGAATGC-3'). The mutagenesis was composed of deletion of the activation peptide and duplication of the tripeptide cleavage site (Fig. 1). Sequence verification of the mutagenesis was performed byideoxycycloterminal nucleotide sequencing (10). The SmaI-EcoRV fragments of the modified factor X cDNA was subcloned into the XbaI site of the expression vector pBN following treatment with Klenow polymerase (11). The expression vector pBN was derived by subcloning the SR-α promoter (12) obtained from pBSI (13) into the NruI-XbaI site of PCR/CMV, thus replacing the CMV promoter with the SR-α promoter. CHO-K1 cells (obtained from the University of California Cell Culture Facility) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 mg/ml penicillin/streptomycin, 2 mM glutamine, and 4 μg/ml vitamin K. The factor X expression vectors pBNX and pBNXa were transfected into CHO cells by the method of lipofectin reagent DNA (Pharmacia LKB Biotechnology Inc.). All chromatographic steps were performed at 4 °C. The column was washed extensively with 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 5 mM CaCl₂, 0.1% bovine serum albumin. Reactions were carried out at room temperature. The solution containing 0.5-1.5 μM factor X, 7.5 μM factor Xa, 20 μM phospholipids, 0.05% human factor X-deficient plasma, and 0.1% bovine serum albumin. Reactions were carried out at room temperature and initiated by adding to the reaction mixture. Aliquots were removed at various times and assayed directly for the production of thrombin. A standard curve of thrombin concentration was constructed. Hydrolysis of the 7-benzamidinohistidine was used to estimate the amount of thrombin generated. For determination of steady-state kinetic constants, the concentration of thrombin was varied between 0.2 and 1.5 μM. Kinetic constants were calculated from the slopes of double-reciprocal plots (16). Each reaction was performed in triplicate. The reported data (Table 1) represent the average of three to five separate determinations.

Functional clotting activities of factors X and Xa were determined using an MLA Electra 800 fibrometer. The time of clotting was determined after addition of bovine factors VII- and X-deficient plasma, rabbit brain cephalin, and factor X or Xa into plasma or factor X-deficient plasma, and actin FS; and (iii) a factor X-dependent prothrombin time assay, which was performed using human factor X-deficient plasma and reagent thromboplastin (17). Clotting assays were performed in duplicate and repeated two to three times, and the activities of factors X and Xa were interpolated from standard clotting curves of purified human factors X and Xa accordingly. An enzyme assay was performed as follows. The concentration of human α-thrombin was determined by active-site titration employing p-nitrophenyl p' guanidinobenzoate HCl (22). This standard α-thrombin preparation was then used to calibrate a reference solution of human antithrombin III (23). Accordingly, human anti-thrombin III titrations of plasma and recombinant human factors X and Xa, based on inhibition of amyloleptic activity (see above), were performed assuming a 1:1 stoichiometry for protease inhibitor (24).

RESULTS

Plasma coagulation factors are unique because of their requirement for several intracellular post-translational modifications including proteolysis of the signal peptide and pro-peptide (Fig. 1, arrows 1-2); γ-carboxylation of glutamate residues in the light chain; and, as in the case of factor X and protein C, intramolecular cleavage of the single chain precursor to the two-chain form (1, 2). During synthesis, proteolytic cleavage of the factor X precursor also occurs at Arg'79-Ser80 (Fig. 1, arrows 3-4) (8, 18, 26). Cleavage at Arg116-Leu117 results in activation (Fig. 1, arrow 5). Following zymogen activation, it is suggested that factor X undergoes autoproteolysis, possibly at Arg124 and Leu125 (Fig. 1, arrow 6) (3, 26). The net effect of this cleavage on factor X enzymatic activity is unknown at present. Signal sequence cleavage most likely occurs at Leu23 and Ser24 (Fig. 1, arrow 1). To combine the intracellular and extracellular domains of factor X, it was essential to cleave the signal sequence from the protein and to prevent further modification of the protein. The extracellular domain of factor X was efficiently cleaved at Arg116-Leu117 as described (24).

W. Church, unpublished data.
vascular processing events that lead to factor X activation into one step, we attempted to express factor X cDNA constructs based on: 1) deletion of the activation peptide and 2) joining of the light and heavy chains by minor modifications of the Arg119-Ser120 intrapeptide cleavage sites. This report details the expression and enzymatic characterization of factor rX and a novel factor, rXa, derived by cellular processing of a unique monomeric precursor.

Human factor X cDNA constructs were transfected into CHO cells, subcloned, and selected for G418-neomycin resistance (Fig. 1). Factor X antigen produced by the transfected stable producing clones was determined by enzyme-linked immunosorbent assay and immunoblotting. Antigen levels averaged ~1 pg/ml for 24-h cultures for both factors rX and rXa. Factors rX and rXa were purified by anion-exchange and immobiloon affinity chromatography. Analysis was made under reducing conditions. Lane 1, 1 μg of plasma factor X; lane 2, 1 μg of RVV-derived plasma factor Xa; lane 3, 1 μg of factor rX; lane 4, 1 μg of factor rXa.

In the factor rXa preparation, sequences for the 35- and 31-kDa species corresponded to the activated plasma factor X heavy chain amino-terminal sequence, and the sequence of the 22-kDa species was comparable to that of the factor rX light chain sequence, with 29% initiating at Val179 and 71% initiating at Ala181. The 43-kDa species did not give reliable amino acid sequence by this procedure. The minor sequences (17 kDa) suggested that proteolytic cleavages had occurred at Arg172-Glu173 and Lys175-Met176 (Fig. 1, arrow 7). Protease inhibitors such as benzamidine or soybean trypsin inhibitor were not used during purification. This could account, in part, for the observed endopeptidase cleavage of the heavy chain by factor Xa and possibly by secreted CHO proteases.

The enzymatic activity of factor rX following activation with RVV was compared with that of RVV-activated plasma factor X (Table I). SDS-PAGE and immunoblot analysis of RVV-activated factor X demonstrated that the single chain precursor content did not change significantly (data not shown). The reduced RVV sensitivity of the factor rX single chain precursor suggests that its participation in the enzymatic activity of factor X in vitro as well as its possible role in vivo are minimal. The catalytic efficiency (k_{cat}/K_m) of factor rX was 50% of that of plasma factor X in the amidolytic assay. This was due to a decrease in k_{cat} and only a slight difference in K_m for recombinant versus plasma factor X. Factor rXa, generated by activation of factor rX with the venom activator and assayed directly without further purification, cleaved prothrombin in the presence of factor Va, phosphatidylcholine/phosphatidylserine vesicles, and Ca^{2+}.

**Fig. 1. Recombinant human factor X constructs.** Deletion mutagenesis of the human factor X activation peptide was performed as described under "Experimental Procedures." rX and rXa refer to the relevant domain arrangement of the recombinant human factor X and Xa precursors. The prepro leader sequence (PrePro), light chain, activation peptide (A.P.), and heavy chain are shown. The sequence modifications to the activation peptide of recombinant human factor Xa are denoted. Amino acid (aa) positions are designated. Arrows denote positions of the known proteolytic cleavage sites.

**Fig. 2. SDS-PAGE analysis of recombinant human factors X and Xa.** Factors rX and rXa were purified by anion-exchange and immobiloon affinity chromatography. Analysis was made under reducing conditions. Lane 1, 1 μg of plasma factor X; lane 2, 1 μg of RVV-derived plasma factor Xa; lane 3, 1 μg of factor rX; lane 4, 1 μg of factor rXa. Factors rX and rXa were purified as described above (see "Experimental Procedures"). Homogeneity of purified factors rX and rXa was determined by SDS-PAGE (Fig. 2). Factor rX (Fig. 2, lane 3) under reducing conditions had three polypeptides, at 75, 45, and 22 kDa. The 45- and 22-kDa species corresponded in migration distance to plasma factor X heavy chain amino-terminal sequence, and the sequence of the 22-kDa species was comparable to that of the factor rX light chain sequence, with 29% initiating at Val179 and 71% initiating at Ala181. The 43-kDa species did not give reliable amino acid sequence by this procedure. The minor sequences (17 kDa) suggested that proteolytic cleavages had occurred at Arg172-Glu173 and Lys175-Met176 (Fig. 1, arrow 7). Protease inhibitors such as benzamidine or soybean trypsin inhibitor were not used during purification. This could account, in part, for the observed endopeptidase cleavage of the heavy chain by factor Xa and possibly by secreted CHO proteases.

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Human Factor X and Xa Expression

Table I

| Factor | $k_m$ | $K_m$ | $k_{cat}/K_m$ |
|--------|-------|-------|---------------|
| Amidolytic assay | | | |
| X | 205.8 ± 2.3 | 150.6 ± 7.0 | 1.4 |
| rX | 136.6 ± 1.7 | 133.7 ± 8.2 | 0.7 |
| Xa | 306.6 ± 1.7 | 133.7 ± 9.6 | 1.9 |
| rXa | 95.1 ± 1.4 | 154.0 ± 5.5 | 0.6 |
| Prothrombinase complex assay | | | |
| X | 17.6 ± 5.0 | 1.1 ± 0.5 | 17.4 |
| rX | 7.5 ± 1.1 | 0.6 ± 0.27 | 14.8 |
| Xa | 9.5 ± 0.4 | 0.27 ± 0.1 | 35.2 |
| rXa | 5.2 ± 0.4 | 0.23 ± 0.1 | 22.6 |

Clotting assay

| Factor | 2PT* activity | APTT activity | PT activity |
|--------|---------------|---------------|-------------|
| rX | 43 ± 3 | 9 ± 3 | 50 ± 10 |
| rXa | 48 ± 7 | | |

* 2PT, two-stage prothrombin time assay; APTT, activated partial thromboplastin time assay; PT, prothrombin time assay.

DISCUSSION

This report describes the expression and characterization of CHO-derived human factors X and Xa. The method chosen to express factor Xa entailed deletion of the activation peptide and duplication of the tripeptide cleavage site (Fig. 1). The modification did not significantly affect the biosynthesis or post-translational processing of the novel precursor. Since factor Xa is capable of being processed in other mammalian cell lines (data not shown), it seems likely that proteolytic cleavage sites, based on minor modifications of native sequences, can be engineered into proteins with some degree of specificity (28).

The novel factor Xa hexapeptide cleavage site shares similarity with the intracellular peptidase endopeptidase Kex2p and carboxypeptidase Kex1p cleavage sites (30, 31) and the recently characterized mammalian FUR gene product (32), which cleaves after paired basic amino acids (PACE) (33). Although it is not known whether these particular proteases are directly involved in the processing of the novel factor Xa monomer precursor, the possibility remains that the unique processing site may be of some utility in other systems.

Attempts to secrete fully active vitamin K-dependent proteins in CHO cells have met with variable success (2, 34). Although prothrombin is expressed in a fully γ-carboxylated and biologically active form, factor IX, protein C, and protein S have been reported to be secreted with incomplete γ-carboxylation (γ-carboxyglutamic acid) and low functional activity. In agreement with CHO-based prothrombin expression, both factors rX and rXa were found to be secreted in biologically active forms (2). However, preliminary γ-carboxylation estimations based on anion-exchange fractionation suggest a mixture of fully and partially γ-carboxylated forms. Work is in progress to further characterize the process and extent of γ-carboxylation in these particular cell lines (29).

Relative to the plasma factors, the lower active enzyme contents of the recombinant factors, 47% for factor rX and 64% for factor rXa, were partially due to a reduction in RVV activation and proteolysis. The reduced RVV conversion could be caused by differing conformations and possible post-translational modifications of factor rXa, affecting both the rate and extent of RVV activation. As mentioned before, factors rX and rXa were not prepared in the presence of protease inhibitors. We have noted that unlike factor rX, factor rXa is less stable in CHO media and is prone to autoproteolysis in purified form. Accordingly, expression of proteolytic inactivated forms of factor rXa is also subject to heavy chain α/β conversion (data not shown). Modifications in cell culture methodologies, purification, and RVV activation should improve the overall specific activities of the recombinant proteins.

This is the first example of the direct expression and characterization of biologically active human factors X and Xa in CHO cells. The ability to express bothzymogen and proteolytically active forms of factor X will greatly facilitate the characterization of functional domains important for activation, complex assembly, substrate association, and catalysis during coagulation. One outcome of this analysis may be the further development of novel coagulation inhibitors useful as antithrombotic agents (25).

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