THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 8, Issue of March 15, pp. 5735-5740, 1993
Printed in U.S.A.

Human Coagulation Factor X Deficiency Caused by a Mutant Signal Peptide That Blocks Cleavage by Signal Peptidase but Not Targeting and Translocation to the Endoplasmic Reticulum*

(Received for publication, July 7, 1992)

Marco Racchi‡, Herbert H. Watzke§, Katherine A. High§, and Mark O. Lively‡¶

From the ‡Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157 and the §Departments of Medicine and Pathology, The University of North Carolina, Chapel Hill, North Carolina 27599

Human factor $X_{Santo\ Domingo}$ is a form of coagulation factor X in which a mutation within the signal peptide region of the precursor protein has been correlated genetically with a severe deficiency of factor X in the affected individual. A point mutation results in substitution of Arg for Gly at the critical −3 position of the factor X signal peptide. To determine the biochemical effect of this mutation on the biosynthesis of factor X, the wild-type and mutant factor X cDNAs were subcloned into a vector for transcription and translation *in vitro*. Translation products of mRNAs encoding portions of both mutant and wild-type proteins were used in a systematic biochemical approach to evaluate directly the effect of the mutation on targeting, transport, and proteolytic processing *in vitro*. The results show that targeting and transport of factor $X_{Santo\ Domingo}$ to the endoplasmic reticulum are functionally dissociated from the removal of the signal peptide by signal peptidase. Factor $X_{Santo\ Domingo}$ is translocated into the endoplasmic reticulum but not processed by signal peptidase. Transient expression of the wild-type and mutant factor X in human embryonic kidney 293 cells revealed apparently normal secretion of the glycosylated two-chain form of factor X but no secretion of factor $X_{Santo\ Domingo}$. Thus, the inability of signal peptidase to cleave factor $X_{Santo\ Domingo}$ is directly responsible for the absence of circulating factor X and leads to the bleeding diathesis in the affected individual.

Secreted proteins are generally synthesized as precursors having NH_2-terminal signal sequences which target nascent secretory proteins to the endoplasmic reticulum (ER)[1] and are then removed by signal peptidase (1). Although signal peptides that target proteins to the ER have widely variable amino acid sequences, they do have three common structural features: a net positive charge at the NH_2 terminus; a central hydrophobic region; and a carboxyl-terminal region with small, nonpolar amino acids at positions −1 and −3 (signal peptides are numbered negatively from the site of cleavage toward the NH_2 terminus of the precursor) preceding the cleavage site (2). A number of mutations within, or near, signal peptides have now been described that alter the processing of human secretory proteins (3–6). Human interferon ω1 has a signal peptide structure that presents two alternative cleavage sites for signal peptidase resulting in circulation of two forms of the protein that differ by two amino acids (7). Additionally, an insertion/deletion polymorphism has been identified in the signal peptide of the human apolipoprotein B gene that predicts two apolipoprotein B signal peptides: one that encodes a peptide of 27 residues and one that encodes a peptide of only 24 residues (8). The effect of this mutation, if any, on secretion of apolipoprotein B has not been demonstrated.

Antithrombin Dublin is an electrophoretically fast variant of antithrombin that has been shown to be the result of a mutation at the −3 position of the signal peptide in which Val is replaced by Glu (4). Individuals expressing the mutation produce a form of antithrombin in which 2 amino acids normally found at the NH_2 terminus have been removed during synthesis. It has been proposed that the Val → Glu substitution redirects the site of cleavage by signal peptidase to a bond 2 amino acid residues toward the COOH terminus of the normal protein. This mutation appears to have no direct correlation with any pathological condition (4).

Similarly, albumin Redhill is an electrophoretically slow form of human serum albumin that contains two different mutations, one of which appears to cause signal peptidase to cleave at an alternate site (3). A substitution of Cys for Arg at the penultimate position of the *pro* peptide (not the signal, or *pre*, peptide) of preproalbumin apparently creates a preferred site for cleavage by signal peptidase. It was hypothesized, but not proven, that this mutation causes signal peptidase to preferentially cleave following the newly introduced Cys, 5 residues into the propeptide. Consequently, albumin Redhill circulates with an additional Arg residue at the NH_2 terminus that would normally have been removed during processing of proalbumin. As with antithrombin Dublin, there is no disease state associated with the presence of albumin Redhill.

Mutations in human signal peptides have been correlated with defective secretion and a consequent pathological state in only two reported cases: preproparathyroid hormone (5),

* This work was supported by National Institutes of Health Grants GM32861 (to M. O. L.) and HL48322 and HL06350 (to K. A. H.). Amino acid sequence analyses were performed in the Protein Analysis Core Laboratory and oligonucleotides were synthesized in the DNA Core Laboratory, each of the Comprehensive Cancer Center of Wake Forest University supported in part by National Institutes of Health Grants CA12197 and RR-04869, as well as a grant from the North Carolina Biotechnology Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157. Tel.: 919-716-4674; Fax: 919-716-7671.

[1] The abbreviations used are: ER, endoplasmic reticulum; HOSP, hen oviduct signal peptidase; FX, coagulation factor X; FXsd, FX Santo Domingo; FXwt, FX wild-type, Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

and coagulation factor $X_{Santo\ Domingo}$ (FXsd; Ref. 6). Substitution of Arg for Cys within the hydrophobic core of preproparathyroid hormone causes a disruption of the core that leads to impaired translocation and/or processing of the nascent hormone. This mutation is the apparent cause of one form of familial isolated hypoparathyroidism (5). FXsd is a mutant form of human factor X in which a point mutation results in the substitution of Arg for Gly at the −3 position of the signal peptide (Fig. 1; Ref. 6). The patient bearing this mutation exhibits a severe bleeding diathesis associated with less than 1% FX enzymatic activity and less then 5% circulating FX protein. The intracellular consequences of this mutation are not immediately predictable since a mutation within a signal peptide could potentially affect secretion at one or more stages. It could block targeting of the nascent polypeptide to the ER or it could block translocation through the bilayer. A mutant signal peptide could also prevent cleavage by signal peptidase or direct cleavage to a new site (9, 10). To distinguish among these possibilities, a systematic approach using mRNAs encoding both mutant and wild-type prepro-FX proteins was used to study the effect of the mutation on targeting, translocation, and proteolytic processing by signal peptidase. The results precisely define the effect of the Santo Domingo mutation and provide a model approach for analyzing the effects of signal peptide mutations on each stage of targeting, translocation, and proteolytic processing of secretory precursor proteins.

EXPERIMENTAL PROCEDURES

Synthesis of mRNA in Vitro—All cloning procedures used restriction enzymes and modifying enzymes purchased from Promega. The cDNAs of prepro-FXwt and prepro-FXsd (6) were subcloned into the *SacI*/*BamHI* sites of the plasmid pGEM-3Zf(+) (Promega) in the correct orientation for transcription by T7 RNA polymerase. Synthesis of mRNA *in vitro* was carried out on 2–5 μg of linearized DNA (specified in figure legends) for 1.5 h at 37 °C in the presence of 40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM each rATP, rCTP, rGTP, and rUTP, and 40 units of T7 RNA polymerase (U. S. Biochemical Corp.).

Polymerase Chain Reaction—Two synthetic oligonucleotides were prepared using an Applied Biosystems Model 380 DNA synthesizer. The sequence of the T7 promoter of the pGEM-3Zf(+) plasmid is: 5′-TAA TAC GAC TCA CTA TA-3′ (oligonucleotide T7). The complementary region between bases 583 and 600 of FX cDNA is: 5′-CGC GGA TCC CTA CCA TGT GAT GCT GTC AGG-3′ (oligonucleotide X200). The underlined sequence is a *BamHI* site added at the 5′-end of oligonucleotide X200. Polymerase chain reaction amplification (11) was performed in a final volume of 100 μl containing 1 μg of target DNA (plasmids pGEM-FXwt/FXsd), 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 μg/ml gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 1 μg of each oligonucleotide, and 1 unit of Taq DNA polymerase (AmpliTaq, Perkin-Elmer Cetus). The reaction mixtures were subjected to 20 cycles of denaturation at 94 °C for 90 s, annealing at 37 °C for 60 s, and primer extension at 72 °C for 70 s. The amplified DNA was recovered by phenol/chloroform extraction and ethanol precipitation and directly transcribed as described above.

Cell-free Protein Synthesis—Translation of mRNA in a cell-free system was performed, according to the manufacturer's (Promega) procedure, in rabbit reticulocyte lysate or wheat germ extract, supplemented with 1 mCi/ml [^{35}S]methionine, 1–2 mCi/ml [^{3}H]isoleucine, or 1–2 mCi/ml [^{3}H]alanine, as specified in the figure legends. Translation products were analyzed by SDS-PAGE (12) in the presence of 2-mercaptoethanol followed by autoradiography of the dried gel using Kodak X-Omat AR films (Eastman Kodak). Enlightning (Du Pont-New England Nuclear) was used for fluorography of gels containing tritiated amino acids.

Amino Acid Sequence Analysis—In preparation for amino acid sequence analysis, the proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) using the transfer conditions as described (13). The dried blot was exposed to film for autoradiography and the bands of interest were cut from the membrane and subjected to automated Edman degradation in an Applied Biosystems Model 475 Protein Sequencer. The content of radiolabel released by each cycle of Edman degradation was determined by liquid scintillation counting.

Signal Peptidase Assay—A translocation-independent assay using full-length and truncated prepro-FXwt/sd proteins (produced by cell-free protein synthesis) as substrates for detergent-solubilized hen oviduct signal peptidase (HOSP) was employed (14). In a total reaction volume of 20 μl, 2–4 μl of cell-free translation products were added to a buffer composed of 10 mM Tris/Cl, pH 8, 250 mM NaCl, 10% (w/v) glycerol, 1 mM $MgCl_2$, 5 mM Chaps, 20 mM dithiothreitol, and 0.08 mg/ml phosphatidylcholine. Next, 5 μl of partially purified HOSP, diluted in the same buffer, were added and the mixture was incubated at 28 °C for 60 min. Reactions were stopped by heating the samples at 100 °C for 3 min in the presence of an equal volume of SDS-PAGE sample buffer, then the reaction products were separated by SDS-PAGE. The dried gel was subjected to autoradiography as described above.

Assays for Translocation, Processing, and Membrane Association—The mRNAs coding for truncated prepro-FXwt/200 and prepro-FXsd/200 were translated in a cell-free rabbit reticulocyte lysate system in the presence of dog pancreas rough microsomes. The procedure followed was essentially as described (10) except that trypsin and chymotrypsin were used at a final concentration of 50 μg/ml each, instead of proteinase K. Carbonate extraction of microsomes was carried out as described (10) except that the translation mixtures were not treated with protease.

Expression in Human Embryonic Kidney Cells—Full-length prepro-FX constructs were transfected into human embryonic kidney 293 cells as described (6). Forty-eight hours following transfection,

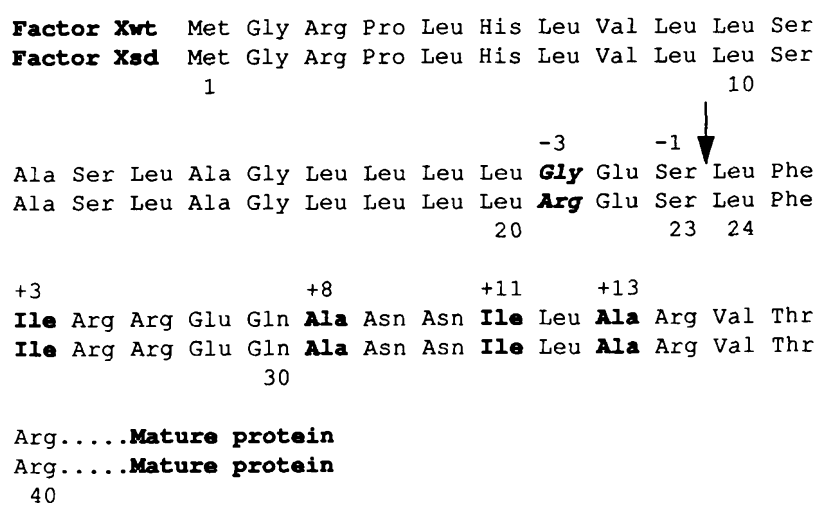

FIG. 1. **The signal peptide sequence of factor X.** The amino acid sequences of the NH_2-terminal prepro region of FXwt (29) and FXsd (6) are shown. The substitution of Arg for Gly at the −3 position of the signal peptide of FXsd is highlighted in *italic* characters. The actual site of signal peptidase cleavage demonstrated in this report (see Fig. 4) is indicated by the *arrow*. *Numbers above* the amino acids indicate their position relative to the signal peptidase cleavage site.

the cells were pulse-labeled for 30 min with 0.5 mCi/ml [^{35}S]methionine in Dulbecco's modified Eagle's medium/F-12 medium in the presence of 10% (v/v) dialyzed fetal bovine serum and then chased for 30 min, 2 h, and 6 h. At each time point, the medium was collected and the cells were lysed in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS. Cell lysate fractions and culture media were immunoprecipitated using rabbit anti-human FX polyclonal antibodies (Dako Corp., Santa Barbara, CA). To determine whether the translated translocated proteins were glycosylated, 5-μl aliquots of immunoprecipitated FX proteins were treated with peptide *N*-glycosidase F (*N*-Glycanase, Genzyme Corp., Cambridge, MA) as described (15). Proteins were separated by SDS-polyacrylamide gel electrophoresis and were detected by autoradiography.

RESULTS

cDNAs encoding both prepro-FX wild-type (prepro-FXwt) and prepro-FX$_{\text{Santo Domingo}}$ (prepro-FXsd) were subcloned into the vector pGEM-3Zf(+) to permit transcription of mRNA for translation *in vitro* (Fig. 2). Proteins produced by cell-free translation of mRNAs can be analyzed in translocation-dependent assays that reconstitute the early steps of the secretory pathway that include membrane targeting, translocation, and proteolytic processing of nascent proteins (16). The same mRNAs can be used to prepare proteins for translocation-independent assays where the fully synthesized precursor proteins are substrates for purified signal peptidase (17). Incubation of full-length secretory precursor proteins with detergent-solubilized HOSP (14) results in cleavage of the signal peptide and the processed protein product can be detected because its smaller size usually results in an increased mobility compared to the precursor when analyzed by SDS-PAGE and autoradiography. Transcription and cell-free translation of the full-length prepro-FXwt and prepro-FXsd mRNAs produced polypeptides each with an apparent molecular mass of 64 kDa based on the mobility during SDS-PAGE (under reducing conditions) although the calculated molecular mass of the polypeptide chain is 54,377 Da. These translation products were immunoprecipitable with anti-human FX antibodies. Pro-FXwt, which was expected to result from removal of the 23-residue signal peptide from full-length prepro-FXwt after treatment with signal peptidase, was not clearly resolved from the precursor by SDS-PAGE under reducing conditions (data not shown) so an alternate approach was taken. Since it has been shown that signal peptidase normally cleaves nascent proteins during synthesis and translocation into the ER before the completion of the polypeptide chain (16), truncated versions of prepro-FX proteins were engineered so that the change in molecular weight resulting from removal of the signal peptide could be readily observed by SDS-PAGE analysis.

The first set of prepro-FX molecules with carboxyl-terminal deletions was created by transcription of prepro-FXwt and prepro-FXsd pGEM plasmids linearized by cleavage at a unique *Pst*I site within the coding region to obtain mRNAs encoding the first 127 amino acids of each protein (Fig. 2). Cell-free translation of these mRNAs yielded proteins migrating on SDS-PAGE with an apparent molecular mass of 18 kDa (Fig. 3*a*, *lanes 1* and *3*). These truncated constructs were designated prepro-FXwt/127 and prepro-FXsd/127 for

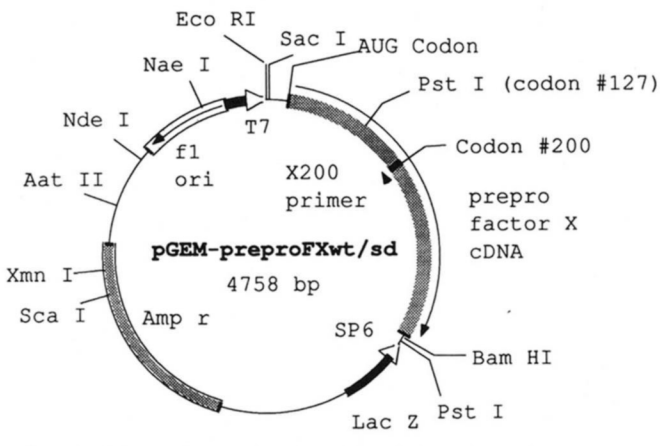

FIG. 2. **Plasmid constructs of full-length and truncated forms of prepro-FXwt/sd.** For the transcription of the full-length mRNAs, the plasmids were linearized with *Bam*HI endonuclease which cleaves the plasmid downstream from the termination codon. To obtain the truncated prepro-FX/127, either wild-type or mutant, the plasmids were linearized with *Pst*I endonuclease at an internal site of the FX cDNA sequence following codon 127. The construction of prepro-FX/200 (wt or sd) was accomplished by polymerase chain reaction amplification of a 712-base pair fragment from the plasmids, which includes the first 200 codons of the FX sequence, as described under "Experimental Procedures." The location of the X200 primer is shown.

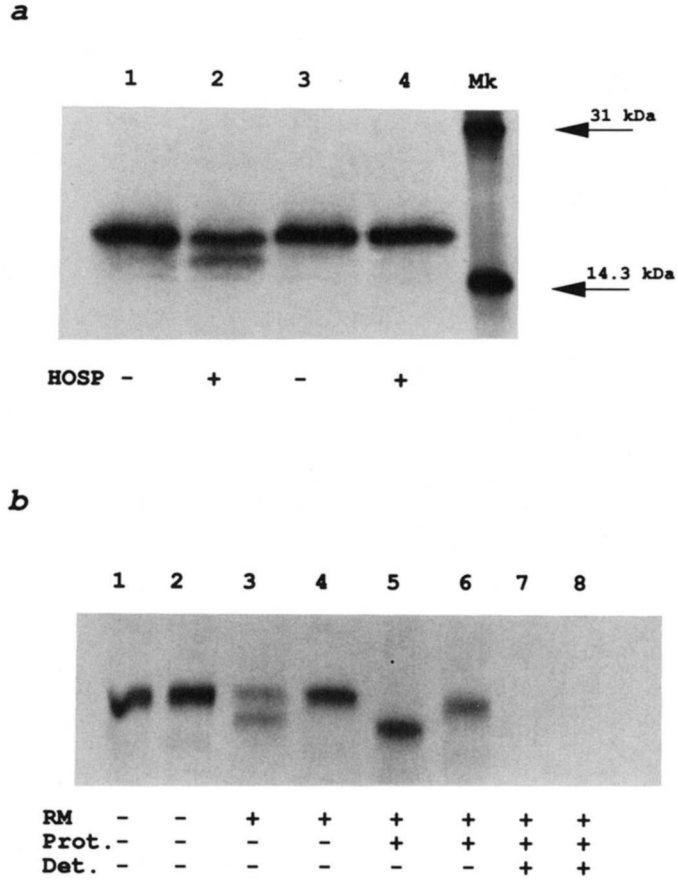

FIG. 3. **Assays for processing, translocation, and segregation of prepro-FXwt/sd.** *a*, translocation-independent processing assays (17) were performed on truncated prepro-FX/127 (wt and sd) obtained by translation of mRNAs in a wheat germ extract system supplemented with 1 mCi/ml [^{3}H]Leu. Aliquots of the translation mixture were subsequently incubated with HOSP (+) or buffer only (−) as described under "Experimental Procedures." *Lanes 1* and *2* show the translation products from the wild-type constructs while *lanes 3* and *4* show the mutant proteins. *b*, for targeting, translocation, and segregation assays, the mRNAs coding for prepro-FX/200 (wt and sd) were translated in a rabbit reticulocyte lysate system supplemented with 1 mCi/ml [^{35}S]Met in the presence (+) or absence (−) of dog pancreas rough microsomes (*RM*). After translation was completed, aliquots of the translation reactions were treated with a mixture of trypsin and chymotrypsin (*Prot.*) in the presence (+) or absence (−) of 1% (v/v) Triton X-100 (*Det.*). *Lanes 1, 3, 5,* and *7* show prepro-FXwt/200; *lanes 2, 4, 6,* and *8* show the mutant protein, prepro-FXsd/200.

the normal and mutant proteins, respectively. The products of cell-free translation were then treated with purified HOSP in a translocation-independent assay to determine the ability of the peptidase to cleave each truncated precursor. HOSP cleaved prepro-FXwt/127 to yield a faster migrating protein band which was consistent with the removal of the signal peptide (Fig. 3*a*, *lane 2*). However, HOSP was unable to cleave prepro-FXsd/127 (Fig. 3*a*, *lane 4*) which differs only by the presence of Arg in place of Gly at the −3 position before the predicted (see below) signal peptidase cleavage site.

To directly determine the site of cleavage by HOSP, prepro-FXwt/127 labeled with either [^{3}H]Ile or [^{3}H]Ala was digested with HOSP. The product of signal peptidase cleavage (Fig. 3*a*, *lane 2*) was isolated and subjected to automated amino acid sequence analysis as described under "Experimental Procedures." Sequence analysis of the signal peptidase cleavage product of [^{3}H]Ile-prepro-FXwt/127 released ^{3}H at cycles 3 and 11 (Fig. 4*A*), whereas the cleavage product of [^{3}H]Ala-prepro-FXwt/127 released ^{3}H at cycle 8 (Fig. 4*B*). These results are consistent with cleavage after Ser^{23} (Fig. 1). In later cycles of sequence analysis of this protein, a larger than usual increase in the carryover of amino acid residues from one cycle to the next accounts for the peaks of radioactivity observed in cycles 12 (Fig. 4*A*) and 9 (Fig. 4*B*). Similar problems were encountered upon sequence analysis of the full-length prepro-FXwt (not shown) and are attributed to the difficulties encountered with the sequence analysis method.

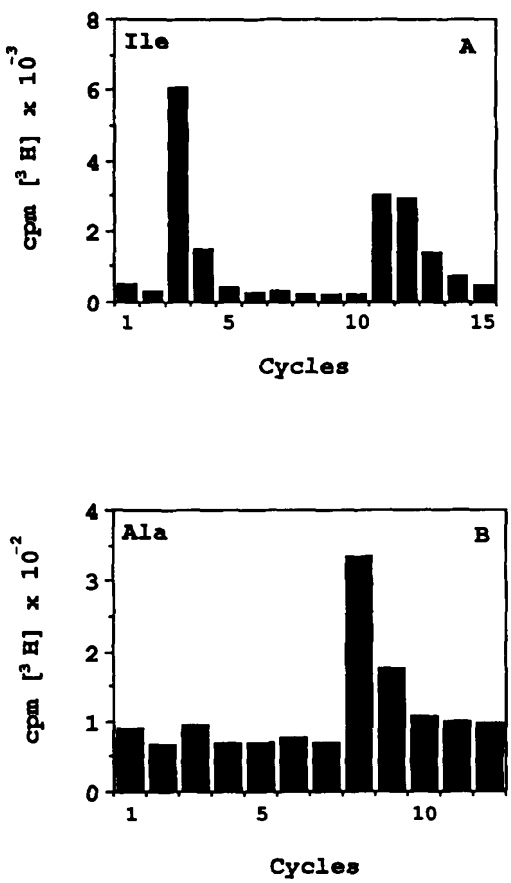

FIG. 4. **Amino acid sequence analysis of the signal peptidase cleavage product of prepro-FXwt/127.** The product of SP cleavage of prepro-FXwt/127 was isolated as described under "Experimental Procedures" and subjected to automated NH_2-terminal amino acid sequence analysis. *Panel A* shows the result of Edman degradation of pro-FX labeled with [^{3}H]Ile. *Panel B* shows the result of amino acid sequence analysis of pro-FX labeled with [^{3}H]Ala.

Having established that the substitution of Arg for Gly blocked translocation-independent cleavage of prepro-FXsd by signal peptidase, we next investigated the effect of the mutation on the membrane targeting and translocation functions of its signal peptide. A second set of truncated molecules, designated prepro-FX/200, was prepared containing the first 200 amino acids of prepro-FX (Fig. 2). Because there were no convenient restriction sites within the coding sequence that would yield a truncation mRNA of the desired size, the polymerase chain reaction (11) was used. Two synthetic oligonucleotides were designed to bracket a 712-base pair region including the T7 RNA polymerase promoter region and the first 200 amino acids of the prepro-FX coding sequence (see "Experimental Procedures"). These oligonucleotides were used as polymerase chain reaction primers to amplify DNA from prepro-FXwt and prepro-FXsd pGEM plasmids. The DNA amplification products were then directly transcribed by T7 RNA polymerase and the resulting mRNAs translated in the cell-free synthesis system. Translation of each truncated mRNA yielded a protein migrating with an apparent molecular mass of 24 kDa (Fig. 3*b*, *lanes 1* and *2*). As with the 127-residue FX molecules, HOSP cleaved prepro-FXwt/200 but not prepro-FXsd/200 in translocation-independent assays (not shown).

Prepro-FX/200 mRNAs were next translated in a cell-free protein synthesis system in the presence of dog pancreas rough microsomes (18) and the locations of the protein products were probed by addition of proteases. Proteins that are targeted to the ER and translocated to the interior of the microsomal vesicles in these translocation-dependent assays are protected from digestion by added proteolytic enzymes which cannot enter the vesicles (19). Prepro-FXwt/200 was cleaved by signal peptidase (Fig. 3*b*, *lane 3*) and the processed form was protected from proteolysis (Fig. 3*b*, *lane 5*). Although not cleaved by signal peptidase (Fig. 3*b*, *lane 4*), prepro-FXsd/200 was properly targeted to the microsomes because its unprocessed form was also protected from digestion by added proteases (Fig. 3*b*, *lane 6*). The protection from proteases observed in each case must have resulted from insertion of prepro-FXsd/200 and pro-FXwt/200 into the microsomes because addition of detergent to the reaction mixture allowed the proteases to penetrate the microsomes and destroy all protected FX molecules, establishing that they were not inherently stable to the protease digestion (Fig. 3*b*, *lanes 7* and *8*).

Following cell-free synthesis of prepro-FX molecules in the presence of microsomes, the vesicles were extracted with 0.1 M Na_2CO_3, pH 11.5, to determine whether the processed protein products were integrated into the lipid bilayer. Treatment of membrane vesicles at high pH solubilizes non-membrane proteins and leaves only integral membrane proteins associated with the sedimentable lipid bilayers (20). This technique revealed that the unprocessed prepro-FXsd/200 and prepro-FXwt/200 were primarily associated with the pelleted membranes (Fig. 5). In contrast to the uncleaved prepro-FXsd/200, the processed pro-FXwt/200 was released into the supernatant upon treatment with carbonate, further demonstrating that the cleaved form of the protein had been correctly targeted to the interior of the microsomes in a soluble state. We conclude that the uncleaved prepro-FXsd/200 is translocated into the microsomal vesicles where it is protected from proteolysis and remains anchored in the ER membrane via the uncleaved hydrophobic signal peptide.

Less than 10% of prepro-FXwt/200 was observed associated

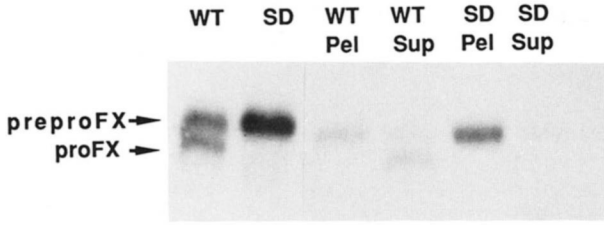

FIG. 5. **Extraction of microsomes with carbonate.** mRNAs coding for prepro-FX/200 wild type (*WT*) and Santo Domingo (*SD*) were translated in a rabbit reticulocyte lysate system supplemented with 1 mCi/ml [^{35}S]Met in the presence of dog pancreas rough microsomes. Following the completion of translation, the microsomes were treated with 0.1 M Na_2CO_3 (Ref. 10). The resulting pellet (*Pel*) and supernatant (*Sup*) fractions from each carbonate extraction were immunoprecipitated with anti-factor X then examined by SDS-polyacrylamide gel electrophoresis and autoradiography.

with the microsome pellet following carbonate extraction (Fig. 5). This precursor form must be exposed on the exterior surface of the microsomes because all uncleaved prepro-FXwt/200 molecules were shown to be susceptible to proteolysis (Fig. 3*b*, *lane 5*). This result suggests that at least a small proportion of correctly targeted precursor protein molecules are present in a membrane-bound form that is not extractable by carbonate yet remains accessible to added proteinases. This may be the result of an experimental artifact of the cell-free protein synthesis system in which the concentration of microsomes was limiting. Nevertheless, the effect of the Santo Domingo mutation on targeting of factor X is clear. In the case of prepro-FXwt/200, all translocated proteins, as defined by protection from proteolysis, are cleaved by signal peptidase. This result stands in contrast to the mutant factor X for which none of the translocated prepro-FXsd/200 molecules were cleaved.

Experiments were next designed to compare the results obtained with the truncated proteins in a cell-free system with the effect of the mutation on the secretion of the full-length proteins transiently expressed in a eukaryotic cell line. We followed the fate of pulse-labeled FX in transfected human embryonic kidney 293 cells (6), in the cell media, and in the intracellular fraction (Fig. 6). The mature, two-chain form of FXwt (21) was secreted beginning at 30 min and reached a maximum level of secretion at approximately 6 h (Fig. 6*a*). In contrast, FXsd was not detected in the cell medium at any time up to 24 h following the pulse of [^{35}S]methionine (Fig. 6*b*). In each case, FXwt and FXsd proteins detected in the intracellular fraction were sensitive to digestion by *N*-glycanase indicating that they were glycosylated (Fig. 7) and therefore had reached the lumen of the ER. Cleavage by *N*-glycanase results in small, measurable increases in electrophoretic mobility of the factor X proteins consistent with the removal of approximately 2 kDa of carbohydrate. These results parallel those obtained in the cell-free system, confirm the efficient translocation of prepro-FXsd into the lumen of the ER, and show the absolute block of the secretion of the mutant protein.

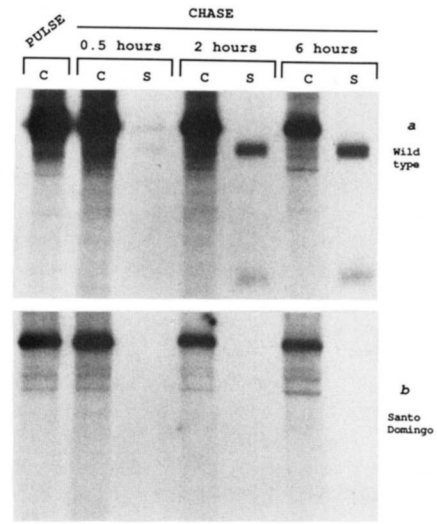

FIG. 6. **Metabolic labeling of cells transfected with prepro-FXwt and with prepro-FXsd.** Human embryonic kidney 293 cells transfected with full-length prepro-FX constructs were pulse-labeled for 30 min with [^{35}S]Met, then chased for 0.5, 2, and 6 h. Cell lysates (*C*) and culture media (*S*) were collected at each time point and immunoprecipitated using rabbit anti-human FX polyclonal antibodies. *Panel a* shows the results for the wild-type FX protein and *panel b* the results for FXsd.

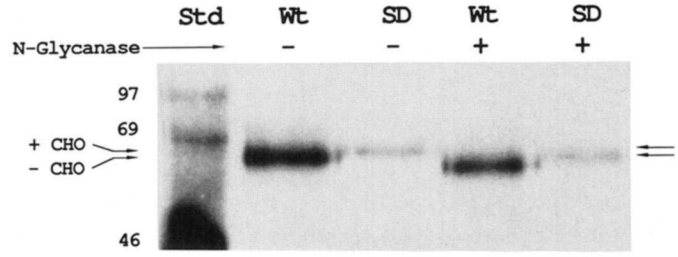

FIG. 7. **Treatment of prepro-FX molecules with *N*-glycanase.** Intracellular forms of factor X proteins produced during metabolic labeling in human embryonic kidney 293 cells were used to determine whether the proteins were glycosylated. The wild-type (*Wt*) and Santo Domingo (*SD*) factor X products present immediately following the chase with unlabeled methionine were either treated (+) or not treated (−) with *N*-glycanase to remove any attached carbohydrate. The products of the *N*-glycanase reaction were then separated by SDS-polyacrylamide electrophoresis and detected by autoradiography. ^{14}C-Labeled molecular weight standards (*Std*) are shown in the *first lane*.

DISCUSSION

Signal peptides are recognized by several different proteins of the eukaryotic translocation and processing apparatus during the initial stages of targeting and transport of nascent proteins into the ER (22). They are apparently recognized first by the 54-kDa subunit of the signal recognition particle which binds to the nascent protein and directs the entire synthetic complex to the ER (23, 24). Once bound to the ER, signal peptides may also interact with components of a postulated translocation apparatus (25). A 35-kDa ER membrane protein has been identified by photochemical cross-linking as a putative signal sequence receptor (26). Additional proteins yet to be identified may interact with the peptide as it is inserted into the translocation site. Finally, most ER signal peptides are proteolytically removed by signal peptidase once the nascent protein has begun the process of transport or insertion into the ER (16). It is difficult to define those aspects of the signal peptide that serve as recognition determinants for the various proteins that bind them because the amino acid sequences of individual signal peptides vary considerably (1, 27). Because the structural characteristics that mediate the specific interactions of signal peptides with the multiple components of the translocation mechanism are not yet well understood, the occurrence of a mutation within a signal peptide can have unpredictable consequences. The experiments described here provide an approach using several established techniques to dissect the early steps of the secretory pathway and identify those affected by mutations in the signal peptide region. The use of truncated mRNAs to produce

shorter precursor protein molecules is especially useful in those cases where the removal of the signal peptide from larger proteins is not easily demonstrated by SDS-PAGE methods.

Since the interaction of the signal peptide with the signal recognition particle is the first critical stage in targeting of the nascent polypeptide chain to the ER, some signal peptide mutants abrogate targeting and cause the protein to be synthesized in the cytoplasm (1). Our experiments demonstrate that the targeting step is not blocked by the mutation in FXsd as the mutant protein is delivered to microsomal vesicles during cell-free protein synthesis (Fig. 3) and to the ER during synthesis in intact cells (Fig. 6). These experiments demonstrate further that the mutation also does not block the process of translocation into the lumen. It is the final step in signal peptide function, its recognition and cleavage by signal peptidase, that is dramatically impaired in the case of FXsd as a direct result of the substitution of Arg for Gly at the −3 position of the FXsd signal peptide. Both truncated forms of prepro-FXwt analyzed in these experiments were cleaved to pro-FX by detergent-solubilized signal peptidase *in vitro*. Under the same experimental conditions, cleavage of the truncated prepro-FXsd molecules was not observed.

Sequence analysis of the signal peptidase cleavage product of prepro-FXwt provided the first direct demonstration of the site of processing of human prepro-FX. A preliminary report on the site of cleavage of the signal peptide for the bovine protein has appeared (28) and the site in human prepro-FX has been correctly inferred from comparison of the gene organization of other vitamin K-dependent coagulation factors (29, 30), but this study is the first to demonstrate the site directly. Signal peptidase cleaved prepro-FX following Ser^{23}.

Among the general structural features that are present in all signal peptides, the amino acids close to the cleavage site appear to have the strongest influence on the specificity of cleavage by signal peptidase (2, 31). Signal peptides must have amino acids with small side chains at the −1 and −3 sites immediately before the site of cleavage (2). Large aromatic, charged, or polar amino acid residues are not observed at these sites. The placement of Arg at the critical −3 position of the prepro-FXsd mutant signal peptide clearly interferes with cleavage. It should be noted that this mutation did not induce a shift in signal peptidase cleavage site, an effect that has been observed in other cases. For example, substitution of Glu for Val at the −3 position of antithrombin Dublin results in redirection of signal peptidase cleavage to a new site 2 residues toward the COOH terminus of the protein (4). Similarly, albumin Redhill is another example in which a mutation appears to redirect signal peptidase cleavage to a new site (3). Signal peptidase appears to have some degree of flexibility in its selection of the site for cleavage if a suitable alternative site is present. In the case of prepro-FXsd, it appears that a suitable alternative cleavage site is not available so the result of the mutation is to block cleavage completely.

The results obtained in the cell-free system were further corroborated by the results of the transient expression of the full-length prepro-FX wild-type and Santo Domingo proteins in human embryonic kidney cells. The wild-type protein was processed and secreted efficiently in its two-chain zymogen form (21) while the mutant FX was never detectable in the cell culture medium. Consistent with the observations *in vitro*, the protein detected in the intracellular compartment was glycosylated demonstrating that the mutant protein was indeed translocated into the ER where glycosylation takes place. The finding that the uncleaved signal peptide anchors prepro-FXsd/200 in the microsomal membrane suggests the possibility that uncleaved prepro-FXsd remains inserted in the ER *in vivo*. As an abnormal ER membrane protein, it is likely that prepro-FXsd is retained in the ER. All membrane or soluble proteins that are retained in the ER because of improper folding, failed oligomerization with required subunits, or aberrant post-translational processing, are eventually degraded (32, 33). Sometimes this degradation process is quite rapid. In the case of prepro-FXsd transfected in human embryonic kidney cells, the protein is relatively stable although it is evident that the protein is degraded slowly in these cells. The patient expressing this mutation did have a very low level of FX antigen detectable in her blood which could represent a small portion of prepro-FXsd that escaped degradation.

It is now clear that mutations in signal peptides of human secretory proteins can have serious consequences. Although only two examples have been described thus far which result in disease, preproparathyroid hormone (5) and factor X Santo Domingo (6), additional examples will surely be recognized in the future. As described here, a systematic approach to the study of the specific effects of such mutations on the earliest stages of the biosynthesis of secretory or membrane proteins will lead to more complete understanding of the role of signal peptides and signal peptidase in human physiology.

Acknowledgments—We thank Dr. Gregory Shelness for the gift of dog pancreas microsomes. We also thank Drs. Shelness, Fred Perrino, and Reidar Wallin for helpful discussions.

REFERENCES

1. Gierasch, L. M. (1989) *Biochemistry* **28,** 923-930
2. von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683-4691
3. Brennan, S. O., Myles, T., Peach, R. J., Donaldson, D., and George, P. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 26-30
4. Daly, M., Bruce, D., Perry, D. J., Price, J., Harper, P. L., O'Meare, A., and Carrell, R. W. (1990) *FEBS Lett.* **273,** 87-90
5. Arnold, A., Horst, S. A., Gardella, T. J., Baba, H., Levine, M. A., and Kronenberg, H. M. (1990) *J. Clin. Invest.* **86,** 1084-1087
6. Watzke, H. H., Wallmark, A., Hamaguchi, N., Giardina, P., Stafford, D. W., and High, K. A. (1991) *J. Clin. Invest.* **88,** 1685-1689
7. Adolf, G. R., Maureu-Fogy, I., Kalsner, I., and Cantell, K. (1990) *J. Biol. Chem.* **265,** 9290-9295
8. Visvikis, S., Chan, L., Siest, G., Drouin, P., and Boerwinkle, E. (1990) *Hum. Genet.* **84,** 373-375
9. Nothwehr, S., and Gordon, J. I. (1989) *J. Biol. Chem.* **264,** 3979-3987
10. Nothwehr, S. F., Hoeltzli, S. D., Allen, K. L., Lively, M. O., and Gordon, J. I. (1990) *J. Biol. Chem.* **265,** 21797-21803
11. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, M. A. (1988) *Science* **239,** 487-491
12. Laemmli, U. K. (1970) *Nature* **227,** 680-685
13. Towbin, H., Starhelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350-4354
14. Baker, R. K., and Lively, M. O. (1987) *Biochemistry* **26,** 8561-8567
15. Stanton, C., and Wallin, R. (1992) *Biochem. J.* **284,** 25-31
16. Blobel, G., and Dobberstein, B. (1975) *J. Cell Biol.* **67,** 835-851
17. Jackson, R. C. (1983) *Methods Enzymol.* **96,** 784-794
18. Walter, P., and Blobel, G. (1983) *Methods Enzymol.* **96,** 84-93
19. Scheele, G. (1983) *Methods Enzymol.* **96,** 94-111
20. Fujiki, Y., Hubbard, A. L., Flowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93,** 97-102
21. DiScipio, R. G., Hermodson, M. A., Yates, S. G., and Davie, E. W. (1977) *Biochemistry* **16,** 698-706
22. Walter, P., Gilmore, R., and Blobel, G. (1984) *Cell* **38,** 5-8
23. Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., and Rapoport, T. A. (1986) *Nature* **320,** 634-636
24. Krieg, U. C., Walter, P., and Johnson, A. E. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8604-8608
25. Walter, P., and Lingappa, V. R. (1986) *Annu. Rev. Cell Biol.* **2,** 499-516
26. Wiedmann, M., Kurzchalia, T. V., Bielka, H., and Rapoport, T. A. (1987) *J. Cell Biol.* **104,** 201-208
27. von Heijne, G. (1985) *J. Mol. Biol.* **184,** 99-105
28. Blanchard, R. A., Faye, K. L. K., and Barnett, J. M. (1985) *Blood* **66** (suppl.) 331a (abstr.)
29. Foster, D. C., Rudinski, M. S., Schach, B. G., Berkner, K. L., Ashok Kumar, A., Hagen, F. S., Sprecher, C. A., Insley, M. Y., and Davie, E. W. (1987) *Biochemistry* **26,** 7003-7011
30. Leytus, S. P., Foster, D. C., Kurachi, K., and Davie, E. W. (1986) *Biochemistry* **25,** 5098-5102
31. Perlman, D., and Halvorson, H. O. (1983) *J. Mol. Biol.* **167,** 391-409
32. Klausner, R. D., and Sitia, R. (1990) *Cell* **62,** 611-614
33. Bonifacino, J. S., and Lippincott-Schwartz, J. (1991) *Curr. Opin. Cell Biol.* **3,** 592-600