Biosynthesis of Prothrombin: Intracellular Localization of the Vitamin K–Dependent Carboxylase and the Sites of γ-Carboxylation

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Prothrombin is a vitamin K–dependent blood coagulation protein that undergoes posttranslational γ-carboxylation and propeptide cleavage during biosynthesis. The propeptide contains the γ-carboxylation recognition site that directs γ-carboxylation. To identify the intracellular sites of carboxylation and propeptide cleavage, we monitored the synthesis of prothrombin in Chinese hamster ovary cells stably transfected with the prothrombin cDNA by immunofluorescent staining. The vitamin K–dependent carboxylase was located in the endoplasmic reticulum and Golgi complex. Antibodies specific to prothrombin processing intermediates were used for immunocytolocalization. Anti-des-γ-carboxyprothrombin antibodies stained only the endoplasmic reticulum whereas antiprothrombin antibodies (specific for the propeptide) and antiprothrombin:Mg(II) antibodies (which bind the carboxylated forms of prothrombin and prothrombin) stained both the endoplasmic reticulum and the Golgi complex. Antiprothrombin:Ca(II)–specific antibodies (which bind only to the carboxylated form of prothrombin lacking the propeptide) stained only the Golgi complex and secretory vesicles, and colocalized with antimanosidase II and anti-p200 in the juxtanuclear Golgi complex. These results indicate that uncarboxylated prothrombin undergoes complete γ-carboxylation in the endoplasmic reticulum and that γ-carboxylation precedes propeptide cleavage during prothrombin biosynthesis.

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abnormal (des-γ-carboxy) prothrombin recognizes uncarboxylated prothrombin and uncarboxylated prothrombin. The conformation-specific antibody antiprothrombin:Mg(II) recognizes fully carboxylated prothrombin regardless of the presence or absence of the propeptide. The conformation-specific antiprothrombin:Ca(II)-specific antibodies bind only to fully carboxylated prothrombin in which the propeptide was cleaved. We conclude that the γ-carboxylase resides in both the endoplasmic reticulum and the Golgi complex, but carboxylation is completed in the endoplasmic reticulum. Complete γ-carboxylation precedes propeptide cleavage during prothrombin biosynthesis.

EXPERIMENTAL METHODS

Antibodies. The preparation of the immunofluorescence-purified conformation-specific antibodies, antiprothrombin:Mg(II) and antiprothrombin:Ca(II)-specific, have been previously described. Anti- abnormal prothrombin antibodies were prepared in rabbits using purified des-γ-carboxy (abnormal) prothrombin as previously described.

Antipropeptide antibodies to the prothrombin propeptide were prepared by the identical methods used to prepare anti-factor IX propeptide antibodies. A synthetic peptide, KGGHVFAPAQQARSL, linking, was synthesized on an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, CA). After purification, the sequence of the peptide was verified by automated Edman degradation using an Applied Biosystems 470A Protein Sequencer. The peptide was coupled to bovine serum albumin (BSA) using glutaraldehyde. The prothrombin propeptide (150 μL; 5 mmol/L), dissolved in phosphate-buffered saline (PBS; 50 mmol/L phosphate, pH 7.4/140 mmol/L NaCl) was mixed with 500 μL of 0.05 mmol/L BSA dissolved in PBS, and 350 μL of PBS was added. Glutaraldehyde (1 mL; 2.5 mmol/L; Sigma, St Louis, MO) was added dropwise to the peptide:BSA solution and stirred for 2 hours at room temperature. The final molar ratio of peptide:BSA:glutaraldehyde was 30:1:100. The peptide conjugate was dialyzed against PBS and stored at −20°C. A New Zealand White rabbit was immunized subcutaneously with 500 μg of the peptide:BSA conjugate in Freund’s complete adjuvant (1 mL). The rabbit subsequently received monthly injections of 200 μg of the peptide:BSA conjugate in Freund’s incomplete adjuvant (1 mL). Antiserum was prepared by the addition of BSA linked to cyanogen bromide-activated Sepharose 4B (Sigma). Approximately 15 mL of antisera was applied to a BSA-Sepharose column (7.5 mg BSA/mL Sepharose; 1 × 5 cm column) at 23°C to remove anti-BSA antibodies. Antibodies that failed to bind were applied to a propeptide-Sepharose column (6 mg of propeptide/mL of Sepharose 4B; 1 × 5 cm column). Bound antibody was eluted with 4 mol/L guanidine HCl, and the guanidine eluate immediately dialyzed against Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, pH 7.4/140 mmol/L NaCl) at 4°C.

Anticarboxylase antibodies directed against bovine carboxypeptidase residues 86-99 and residues 661-675 were prepared as described. The IgG fraction of this antiserum was purified by affinity chromatography using protein A-Sepharose (Sigma).

Expression and purification of prothrombin:R-2D,R-1E. Mutant cDNA for prothrombin:R-2D,R-1E was constructed using the site overlap extension method of the polymerase chain reaction (PCR). After gel purification and restriction digestion, the mutagenic DNA fragment was substituted for the wild-type fragment in pMT2-PT, a mammalian expression vector containing the cDNA for human prothrombin and the gene for dihydrofolate reductase. The entire region generated by the PCR was sequenced before transfection. A clonal population of dihydrofolate reductase-deficient CHO cells, CHO Dux-B11/4, was further selected for its ability to fully carboxylate prothrombin. Plasmid DNA was transfected into the cells using lipofectin (GIBCO-BRL Life Technologies, Gaithersburg, MD). Clones expressing prothrombin were detected by a filter hybridization assay using polyclonal antiprothrombin antibodies. CHO cells producing the highest levels of the mutant prothrombin were introduced in 2,000 μL of medium into Nunc cell factories and allowed to grow for 7 days. The collected medium was concentrated in an Amicon Hollow Fiber Concentrator (model RA2000; Beverly, MA) and applied to an immunofluorescence column of polyclonal antiprothrombin antibodies. The column was washed with boreate-buffered saline (1 mol/L NaCl, 0.1 mol/L boric acid, pH 8.0) containing 2 mmol/L benzamidine and 0.1% Tween 80. The column was washed with TBS (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) before elution. The protein was eluted with 2 mol/L GuHCl, dialyzed three times against TBS containing 1 mmol/L benzamidine, and concentrated using Amicon Centriprep 30 concentrators.

Immunochemistry. A CHO cell line expressing prothrombin was grown to near confluence on glass coverslips (Corning Glass, Corning, NY) in selective media (AdT deficient) containing 10 μg/mL of vitamin K1. The coverslips were transferred to ceramic boats submerged in TBS and the cells were fixed at 25°C by incubating the coverslips for 20 minutes in 3.7% formaldehyde (Fisher, Pittsburgh, PA) diluted into TBS containing either 3 mmol/L CaCl2 or 3 mmol/L EDTA. After washing the coverslips in TBS, cells were permeabilized by incubating the coverslips at 25°C for 30 minutes in 0.5% Triton X-100 (Sigma) in TBS. After washing with TBS, coverslips were fixed at 4°C in TBS/0.1% BSA/0.5% NaN3, Antibody (10 μL at 5 to 100 μg/mL in 0.2% ovalbumin/TBS/0.5%NaN3) was applied to the cells on the coverslips, and the coverslips incubated for 45 minutes at 37°C. After washing in TBS, the cells were stained with rhodamine-labeled goat-antirabbit IgG (Pierce, Rockford, IL). After washing in TBS, the coverslips were mounted on glass slides with Airvol (Air Products, Allentown, PA). Immunofluorescence microscopy was performed on a Zeiss Axioscope fluorescence microscope at 630× magnification (Zeiss, Thornwood, NY). Micrographs were prepared using Kodak T-400 black and white film (Eastman Kodak, Rochester, NY).

For double-label experiments, rabbit antiprothrombin:Ca(II)-specific antibodies (100 μg/mL) and AD7 (a murine anti-p200 MoAb; ascites fluid 1:100 dilution) were mixed in dilution buffer (2% BSA, TBS; pH 7.4, 0.02% NaN3) applied to coverslips together, and incubated as described above. After washing with PIPES buffer (15 mmol/L PIPES, pH 7.4, 140 mmol/L NaCl, 1 mmol/L CaCl2), the coverslips were incubated with a mixture of rhodamine-conjugated goat-antimouse IgG (Pierce; diluted 1:250 into dilution buffer) and fluorescein-conjugated donkey-antirabbit IgG (Pierce; diluted 1:100 in dilution buffer). In control experiments, either rabbit antiprothrombin:Ca(II) antibodies, AD7, or both were omitted to evaluate the specificity of staining with the secondary antibody. Samples were washed again in PIPES buffer and photographed at 630× magnification using a Zeiss Axioscope fluorescence microscope. Filter sets were used for excitation and visualization of fluorescein and rhoda-
BIOSYNTHESIS OF PROTHROMBIN

Fig 1. Purification and characterization of prothrombin. (A) SDS gel electrophoresis of prothrombin and proprothrombin forms. Lane 1, des-γ-carboxy-proPT/R-2D,R-1E; lane 2, proPT/R-2D,R-1E; lane 3, plasma-derived prothrombin. Proteins were visualized with Coomassie blue. (B) Western blot of proprothrombin and prothrombin. Lane 1, plasma-derived prothrombin; lane 2, proPT/R-2D,R-1E; lane 3, des-γ-carboxy-proPT/R-2D,R-1E; lane 4, plasma-derived prothrombin; lane 5, proPT/R-2D,R-1E; lane 6, des-γ-carboxy-proPT/R-2D,R-1E. Lanes 1 through 3 were developed with anti-thrombin:total antibodies. Lanes 4 through 6 were developed with anti-proPT antibodies. The blots depicted in lanes 1 through 3 and lanes 4 through 6 were performed separately with their respective antibodies.

Fig 2. Interaction of antiprothrombin antibodies with prothrombin, proPT/R-2D,R-1E, and des-γ-carboxyprothrombin. (A) Anti-proPT antibodies directed against the propeptide. (B) Anti-abnormal prothrombin antibodies. (C) Antiprothrombin:Mg(II) antibodies. (D) Antiprothrombin:Ca(II)-specific antibodies. (■), Prothrombin; (△), proPT/R-2D,R-1E; (▲), des-γ-carboxyprothrombin; (◇), des-γ-carboxy-proPT/RD-2,RE-1.

RESULTS

Purification and characterization of antibodies directed against the prothrombin propeptide. We generated a polyclonal antibody directed against the prothrombin propeptide to examine the presence or absence of the propeptide in various intracellular prothrombin biosynthetic intermediates. This antibody was prepared in a rabbit using a synthetic peptide based on the sequence of prothrombin (proPT) (residues -18 to -1) that was coupled to BSA as a carrier protein. The antiprothrombin propeptide antibodies were purified from rabbit antiserum by sequential immunoaffinity chromatography. Antibody that failed to bind to BSA was applied to a propeptide-Sepharose column. The
antibody that bound was eluted with 4 mol/L guanidine HCl and is referred to as anti-proPT antibody.

Expression and characterization of proPT/R-2D,R-1E. Prothrombin is efficiently processed in heterologous CHO cells\(^1\) and no prothrombin can be detected after secretion. To obtain prothrombin, CHO cells were transfected with a mutated form of prothrombin cDNA. These cells expressed the mutant form of prothrombin, proPT/R-2D,R-1E in which the arginine at -2 was mutated to an aspartic acid and the arginine at -1 that is adjacent to the propeptide cleavage site was mutated to a glutamic acid. Cells were grown in the presence of vitamin K to obtain prothrombin that contained \(\gamma\)-carboxyglutamic acid; cells were grown in the presence of warfarin, a vitamin K antagonist, to obtain prothrombin that was deficient in \(\gamma\)-carboxyglutamic acid. This mutant prothrombin is similar to the naturally occurring mutant, factor IX Cambridge,\(^4\) in which factor IX circulates with an 18-residue propeptide N-terminal extension. ProPT/R-2D,R-1E, expressed in CHO cells in the presence of vitamin K or in the presence of warfarin, migrated as single bands after sodium dodecyl sulfate (SDS) gel electrophoresis (Fig 1A). Direct \(\gamma\)-carboxyglutamic acid analysis of the alkaline hydrolysate showed that proPT/R-2D,R-1E expressed in CHO cells in the presence of vitamin K contained 8.2 ± 0.4 mol of \(\gamma\)-carboxyglutamic acid per mol of protein; given the presence of minor contaminants, the prothrombin precursor is likely fully carboxylated. In contrast, proPT/R-2D,R-1E expressed in CHO cells in the presence of warfarin had no detectable \(\gamma\)-carboxyglutamic acid. Upon Western blot analysis, both the carboxylated and the acarboxy form of prothrombin/R-2D,R-1E as well as prothrombin stained with antiprothrombin:total antibodies (Fig 1B). However, the carboxylated and the acarboxy form of proPT/R-2D,R-1E stained with anti-proPT antibodies but prothrombin, lacking the propeptide, did not (Fig 1B). These results indicate that purified prothrombin, in both its carboxylated and its uncarboxylated form, contains the propeptide and is reactive with anti-proPT antibodies, in contrast to prothrombin.

Specificity of the antiprothrombin antibodies. Although we have previously established the specificity of antibodies for various synthetic prothrombin intermediates\(^5\) and parallel studies with factor IX antibodies allowed us to anticipate certain results for prothrombin,\(^6\) the absence of prior studies on prothrombin necessitated evaluation of the interaction of antipropeptide antibodies, antiabnormal prothrombin antibodies, antiprothrombin:Mg\(\text{II}\) antibodies, and antiprothrombin:Ca\(\text{II}\)-specific antibodies with prothrombin, proPT/R-2D,R-1E, des-\(\gamma\)-carboxyprothrombin, and des-\(\gamma\)-carboxyproPT/R-2D,R-1E. The interaction of each antibody population was studied using a competition radioimmunoassay. Using \(^{125}\text{I}-\)labeled proPT/R-2D,R-1E, the interaction of \(^{125}\text{I}-\)labeled proPT/R-2D,R-1E with anti-proPT antibodies was inhibited with proPT/R-2D,R-1E and des-\(\gamma\)-carboxyproPT/R-2D,R-1E but not prothrombin or des-\(\gamma\)-carboxyprothrombin (Fig 2A). Using \(^{125}\text{I}-\)labeled des-\(\gamma\)-carboxyprothrombin, the interaction of \(^{125}\text{I}-\)labeled des-\(\gamma\)-carboxyprothrombin with antiabnormal prothrombin antibodies was inhibited with des-\(\gamma\)-carboxyprothrombin and, to a lesser extent, by des-\(\gamma\)-carboxyproPT/R-2D,R-1E, but not prothrombin (Fig 2B). ProPT/R-2D,R-1E exhibited minimal reactivity with this antibody. The interaction of \(^{125}\text{I}-\)labeled des-\(\gamma\)-carboxyprothrombin with antiabnormal prothrombin antibodies was inhibited with des-\(\gamma\)-carboxyprothrombin and, to a lesser extent, by des-\(\gamma\)-carboxyproPT/R-2D,R-1E, but not prothrombin (Fig 2B). ProPT/R-2D,R-1E exhibited minimal reactivity with this antibody. The interaction of \(^{125}\text{I}-\)labeled des-\(\gamma\)-carboxyprothrombin with antiabnormal prothrombin antibodies was inhibited with des-\(\gamma\)-carboxyprothrombin and, to a lesser extent, by des-\(\gamma\)-carboxyproPT/R-2D,R-1E, but not prothrombin (Fig 2B).
BIOSYNTHESIS OF PROTHROMBIN

Fig 4. Localization of fully carboxylated prothrombin species. CHO cells expressing prothrombin were stained with antibodies, as indicated. (A) Antiprothrombin:Mg(II) antibodies; (B) antiprothrombin:Mg(II) antibodies, cells were fixed in the presence of EDTA; (C) antiprothrombin:Ca(II)-specific antibodies; (D) antiprothrombin:Ca(II)-specific antibodies; cells were fixed in the presence of EDTA. The arrowheads identify the juxtanuclear Golgi complex.

labeled prothrombin with antiprothrombin:Mg(II) antibodies was inhibited with proPT/R-2D.R-1E and prothrombin but not des-γ-carboxyprothrombin or des-γ-carboxyproPT/R-2D.R-1E (Fig 2C). Using 125I-labeled prothrombin, the interaction of 125I-labeled prothrombin with antiprothrombin:Ca(II)-specific antibodies was inhibited with prothrombin but proPT/R-2D.R-1E, des-γ-carboxyproPT/R-2D.R-1E and des-γ-carboxyprothrombin were unreactive with this antibody (Fig 2D). These results indicate that anti-proPT antibodies can be used to identify species that contain the propeptide, regardless of the state of carboxylation; that antiabnormal prothrombin antibodies can be used to identify

Fig 5. Intracellular localization of the juxtanuclear Golgi complex. Immunofluorescent staining of mannosidase II in CHO cells expressing prothrombin. Mannosidase II, a marker of the juxtanuclear Golgi complex, was stained with rabbit anti-mannosidase II antibodies.
Antiprothrombin:total antibodies bind to prothrombin regardless of the carboxylation state or the presence of the propeptide. Antiprothrombin:total antibodies stain organelles in these cells, including the meshlike endoplasmic reticulum and the perinuclear Golgi complex (data not shown). CHO cells transfected with the expression vector lacking prothrombin cDNA were not reactive with antiprothrombin:total antibodies. Similarly, an irrelevant antibody, anti-factor IX:total, failed to stain CHO cells expressing prothrombin nor did the fluorescent antibody alone interact with cells (data not shown). Furthermore, if CHO cells expressing prothrombin were not permeabilized before immunofluorescent staining with antiprothrombin:total antibodies, no staining was evident (data not shown). Thus, these results indicate that these antibodies specifically bind to intracellular forms of prothrombin.

Localization of acarboxy forms of prothrombin. Figure 3A shows the staining with anti-abnormal prothrombin antibodies of cells synthesizing prothrombin. These antibodies react with uncarboxylated or poorly carboxylated prothrombin species regardless of the presence or absence of the propeptide. The endoplasmic reticulum is stained specifically, whereas no staining of the Golgi complex can be observed. These results indicate that at the expression levels characteristic of these stably transfected CHO cells, γ-carboxylation is an efficient process and appears to be completed in the endoplasmic reticulum.

Using antitbovine carboxylase antibodies to stain the endogenous γ-carboxylase of CHO cells, the endoplasmic reticulum and the Golgi complex represent the major sites of carboxylase (Fig 3C). An endoplasmic reticulum marker, BiP (HSP78), stained with anti-BiP antibodies (Fig 3D). These results indicate that the carboxylase resides in both the endoplasmic reticulum and the Golgi complex. However, carboxylation appears complete before transit of prothrombin from the endoplasmic reticulum to the Golgi complex because no des-γ-carboxyprothrombin is observed in the Golgi complex.

Localization of propeptide-containing forms of prothrombin. Anti-proPT antibodies reacted with prothrombin regardless of the degree of γ-carboxylation, but did not bind to prothrombin or des-γ-carboxyprothrombin. As shown in Fig 3B, these antibodies stain both the endoplasmic reticulum and a component of the Golgi complex. The Golgi staining in CHO cells is perinuclear, but the particular region of the Golgi cannot be determined from these light micrographs. Because these antibodies do not bind to the free propeptide (J.A.B., unpublished results, December 1990), interpretation of these micrographs is not complicated by the localization of cleaved propeptide.

Localization of fully carboxylated forms of prothrombin. Antiprothrombin:Mg(II) antibodies bind to the fully carboxylated form of prothrombin regardless of the presence or absence of propeptide. These antibodies bind prothrombin only in the presence of metal ions. As shown in Fig 4A, antiprothrombin:Mg(II) antibodies are reactive with antigen in the endoplasmic reticulum and in the Golgi complex. When cells were fixed in the presence of EDTA, this binding was abolished (Fig 4B). When CHO cells were cultured in

Fig 6. Colocalization of fully carboxylated prothrombin and p200 to the Golgi complex. Affinity-purified rabbit antiprothrombin:Ca(II)-specific polyclonal antibodies and AD7, a murine anti-p200 MoAb, were used in double-label immunofluorescence experiments as detailed in Experimental Methods. (A) Carboxylated prothrombin lacking the propeptide; (B) p200.

des-γ-carboxylated prothrombin species, regardless of the presence or absence of the propeptide; that antiprothrombin:Mg(II) antibodies recognize fully carboxylated prothrombin, regardless of the presence or absence of propeptide; that antiprothrombin:Ca(II) antibodies recognize fully carboxylated prothrombin only after the propeptide has been cleaved.

Immunofluorescent staining of CHO cells expressing prothrombin. Antiprothrombin:total antibodies bind to prothrombin regardless of the carboxylation state or the presence of the propeptide. Antiprothrombin:total antibodies stain organelles in these cells, including the meshlike endoplasmic reticulum and the perinuclear Golgi complex (data not shown). CHO cells transfected with the expression vector lacking prothrombin cDNA were not reactive with antiprothrombin:total antibodies. Similarly, an irrelevant antibody, anti-factor IX:total, failed to stain CHO cells expressing prothrombin nor did the fluorescent antibody alone interact with cells (data not shown). Furthermore, if CHO cells expressing prothrombin were not permeabilized before immunofluorescent staining with antiprothrombin:total antibodies, no staining was evident (data not shown). Thus, these results indicate that these antibodies specifically bind to intracellular forms of prothrombin.
BIOSYNTHESIS OF PROTHROMBIN

Fig 7. Schematic summary of the biosynthesis of prothrombin. Prothrombin is synthesized in the endoplasmic reticulum (ER) as a precursor protein (acarboxyproPT), including a propeptide N-terminal extension that contains the γ-carboxylation recognition site. This precursor protein is not carboxylated. Although the carboxylase (C) resides in both the endoplasmic reticulum and the Golgi complex, carboxylation is complete before the protein leaves the endoplasmic reticulum. Fully carboxylated prothrombin (ProPT) is present in both the endoplasmic reticulum and in the Golgi complex. The propeptide is cleaved by furin, a protease of the trans Golgi network. Prothrombin, fully carboxylated and lacking the propeptide, appears only in the juxtanuclear Golgi complex and in secretory granules (SV) that represent the final step in the constitutive biosynthesis of prothrombin. (C), Carboxylase; (0), Bip; (B), p200; (M), mannosidase II; (G), furin; PM, plasma membrane. Propeptide (rectangular appendage), reactive with anti-proPT antibodies; uncarboxylated glutamic acids (I), define antigen reactive with antianomalous prothrombin; γ-carboxyglutamic acid (Y), define antigen reactive with antiprothrombin:Mg(II) antibodies in the presence or absence of propeptide and define antigen reactive with antiprothrombin:Ca(II)-specific antibodies only in the absence of propeptide.

medium containing warfarin to inhibit γ-carboxylation, they failed to interact with antiprothrombin:Mg(II), even in the presence of calcium ions (data not shown). These results confirm that the interaction of these antibodies with prothrombin species requires γ-carboxylation and that carboxylated prothrombin and carboxylated proprothrombin reside in both the endoplasmic reticulum and the Golgi complex.

Localization of fully carboxylated forms of prothrombin that lack the propeptide. Antiprothrombin:Ca(II)-specific antibodies bind to the fully carboxylated form of prothrombin that lacks the propeptide. These antibodies bind prothrombin only in the presence of Ca(II) ions and not other divalent metal ions. As shown in Fig 4C, antiprothrombin:Ca(II)-specific antibodies are reactive with antigen in small granules within the cytoplasm as well as the Golgi complex. In the presence of EDTA, this binding was abolished (Fig 4D).

The juxtanuclear Golgi complex was localized using polyclonal antibodies to mannosidase II (Fig 5); mannosidase II is concentrated in the medial Golgi compartment,30 but this compartment and the trans Golgi network cannot be distinguished by immunofluorescence techniques. To simultaneously localize prothrombin and the Golgi complex, we performed a double-label experiment to visualize carboxylated prothrombin, using antiprothrombin:Ca(II)-specific antibodies, and p200, a Golgi complex marker that colocalizes with mannosidase II, using the MoAb AD7.26 p200 and carboxylated, propeptide-free prothrombin colocalized to the juxtanuclear Golgi complex and small cytoplasmic granules (Fig 6). In control experiments, omission of AD7 resulted in the absence of a rhodamine signal, omission of antiprothrombin:Ca(II)-specific antibodies resulted in the absence of a fluorescein signal, and omission of both AD7 and antiprothrombin:Ca(II)-specific antibodies resulted in the absence of both rhodamine and fluorescein signals. Thus, mature prothrombin is located in the juxtanuclear Golgi complex and in small secretory granules.

DISCUSSION

The biosynthesis of the vitamin K-dependent proteins includes the posttranslational synthesis of γ-carboxyglutamic acid. In this study we have identified the site of γ-carboxylation and the site of propeptide cleavage by tracking immunochemically distinct forms of prothrombin through subcellular organelles. Using specific antibodies, we have been able to identify four chemically distinct prothrombin species: (1) uncarboxylated proprothrombin; (2) carboxylated proprothrombin; (3) uncarboxylated prothrombin; and (4) carboxylated prothrombin, the mature and biologically active species that circulates in blood. We and others have previously shown that the vitamin K-dependent proteins lack biologic activity if the propeptide is not cleaved4,5,31 or if carboxylation is not complete.32,33 Such forms lack the ability to bind to membrane surfaces in the presence of calcium.

We demonstrate by direct immunofluorescence studies that the carboxylase resides in the endoplasmic reticulum and the Golgi complex. Previously, Carlisle and Suttie34 had identified the endoplasmic reticulum as the site of carboxylase activity in studies based on the preparation and assay of
carboxylase in disrupted bovine liver cells. Wallin et al extended these studies by isolating preparations of both endoplasmic reticulum and the Golgi complex; both contained carboxylase activity. Because there remains uncertainty about the purity of these subcellular fractions, we used anti-carboxylase antibodies in situ to perform immunocytochemical localization. These antibodies, including anti-carboxylase 86–99 and anti–carboxylase 661–675, were prepared against peptides based on the sequence of bovine carboxylase. Both reagents showed identical staining patterns of both the endoplasmic reticulum and the Golgi complex. Thus, the vitamin K–dependent carboxylase is resident in both the endoplasmic reticulum and the Golgi complex. However, the apparent absence, within experimental limits, of uncarboxylated prothrombin in the Golgi complex suggests that prothrombin is efficiently carboxylated in the endoplasmic reticulum before transit to the Golgi complex. The presence of carboxylase in the Golgi complex may be caused by membrane fusion and recycling of the carboxylase back to the endoplasmic reticulum, although the carboxylase lacks any of the known recognition elements that localize membrane proteins in the endoplasmic reticulum.

As previously described for factor IX, a synthetic propeptide based on the sequence of prothrombin was prepared and, after conjugation to albumin, was used as immunogen to generate rabbit antiprothrombin propeptide antibodies. After immunoaffinity purification, these antibodies were not reactive with prothrombin, but were reactive with the mutant prothrombin, proPT/R-2D,R-1E, that we used as a model for the propeptide-containing prothrombin species. In contrast to the expression of recombinant factor IX CHO cells, where about 7% of the factor IX generated is profactor IX, CHO cells expressing prothrombin cleave the propeptide efficiently and no prothrombin could be detected. Thus, we mutated residues –2 and –1 so that the propeptide cleavage site was altered and the propeptide remained attached to prothrombin. ProPT/R-2D,R-1E was nearly fully carboxylated and yielded a single band upon analysis by SDS gel electrophoresis. It was reactive with anti-proPT antibodies, in contradistinction to prothrombin. Thus, the identification of prothrombin within the cell by anti-proPT antibodies is based on the expression of antigenic determinants in wild-type prothrombin and prothrombin with residues –1 and –2 altered.

This in situ analysis illustrates a pathway for the biosynthesis of the vitamin K–dependent proteins (Fig 7). Prothrombin is synthesized in the endoplasmic reticulum as a precursor protein, including a propeptide N-terminal extension that contains the γ-carboxylation recognition site. This precursor protein, prothrombin, does not contain γ-carboxyglutamic acid. This is consistent with carboxylation as a posttranslational process rather than a cotranslational process. Proprothrombin undergoes subsequent γ-carboxylation. Although the carboxylase resides in both the endoplasmic reticulum and the Golgi complex, carboxylation is essentially complete before the protein leaves the endoplasmic reticulum. Thus, fully carboxylated prothrombin exists within the cell, and is present within both the endoplasmic reticulum and in the Golgi complex. This result confirms our original hypothesis that the carboxylation recognition site on the propeptide directs γ-carboxylation, and that propeptide cleavage must follow γ-carboxylation. Fully carboxylated prothrombin is further processed in the Golgi complex, with cleavage of the propeptide. Proprothrombin contains the furin consensus sequence. Because furin is known to reside in the trans Golgi network, and by immunofluorescence microscopy colocalizes to the Golgi complex with mannosidase II, our observation of the conversion of proprothrombin to prothrombin in a Golgi compartment is consistent with the function and location of this enzyme. Prothrombin, fully carboxylated and lacking the propeptide, appears only in the Golgi complex and in secretory vesicles for constitutive secretion of this protein into the blood where it circulates as a plasma protein.

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BIOSYNTHESIS OF PROTHROMBIN

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Biosynthesis of prothrombin: intracellular localization of the vitamin K-dependent carboxylase and the sites of gamma-carboxylation

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