Intracellular maturation of the γ-carboxyglutamic acid (Gla) region in prothrombin coincides with release of the propeptide

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Vitamin K-dependent coagulation factors undergo several post-translational modifications before the proteins are secreted into the blood as functional zymogens of the coagulation system. The modifications include Asn-linked glycosylation, Asn/Asp hydroxylation, removal of a signal peptide for translocation of the polypeptide into the endoplasmic reticulum and removal of a propeptide which, when attached to the intracellular coagulation factor precursor, directs the protein for vitamin K-dependent γ-carboxylation. γ-Carboxylation of targeted Glu residues results in formation of Ca^{2+}-binding γ-carboxyglutamic acid (Gla) residues. Ca^{2+} binding by these residues induces a conformational change in the protein which is a necessary event for optimal activation or activity of the clotting factor in blood. In the present study we have monitored the intracellular prothrombin precursor in the secretory pathway of liver cells to determine the effect that the propeptide has on Ca^{2+}-dependent folding of the protein. The data provide evidence that the Ca^{2+}-induced conformational change required for activation of prothrombin coincides with release of the propeptide in the trans-Golgi apparatus of the liver cell and elucidates an important function for the endoproteinase furin in biosynthesis of vitamin K-dependent clotting factors.

INTRODUCTION

Vitamin K-dependent coagulation factors are Asn-linked secretory glycoproteins that are synthesized by the liver [1–4]. In early processing by the hepatocyte, these proteins contain a 16–18-residue propeptide attached to the amino acid appearing at the N-terminus in the mature plasma protein [2]. The propeptide is the recognition element that directs these precursors for γ-carboxylation by the vitamin K-dependent carboxylase. The carboxylase, an integral membrane protein of the endoplasmic reticulum, converts 10–12 Glu residues in the N-terminal part of the proteins to Ca^{2+}-binding γ-carboxyglutamic acid (Gla) residues [2,3]. Binding of Ca^{2+} to the Gla domain of these proteins induces a conformational change in the domain which is necessary for optimal activation or activity of the vitamin K-dependent clotting factors [1,5]. Correct Ca^{2+}-dependent folding of the Gla domain has been shown to be dependent upon γ-carboxylation of all targeted Glu residues defining the Gla domain [6,7]. It has also been documented that the propeptide sequence harbours the information that dictates the efficacy and extent of γ-carboxylation of the Gla region [8].

Incompletely γ-carboxylated mutant forms of clotting factor IX have been described which circulate in the plasma of haemophilia B subjects with the propeptide still attached [9,10]. These proteins do not attain optimal activity because of incomplete folding of the Gla domain. In factor IX Cambridge [9] and factor IX San Dimas [10], mutations in the propeptide sequence have been documented at positions −1 (Arg to Ser) and −4 (Arg to Gln) respectively. These basic amino acid residues constitute parts of a recognition site targeted by the endoproteinase furin, an enzyme which cleaves the propeptide from the precursor before the coagulation factor enters the circulating blood [11]. Furin is a Ca^{2+}-dependent subtilisin-like endoproteinase that is located in the trans-Golgi apparatus of the secretory pathway [12,13], and the enzyme has been shown to be involved in proteolytic processing of a variety of secretory proteins destined for constitutive secretion from the cell [13]. Since no information is available regarding the extent to which the propeptide itself interferes with folding of the Gla domain, we have carried out experiments at the cellular level to acquire this information. Conformation-specific antibodies raised against the Ca^{2+}-induced Gla domain in plasma prothrombin were used to follow the prothrombin precursor on its 'journey' through the secretory pathway of the liver cell. Our data demonstrate that release of the propeptide by furin in the trans-Golgi apparatus is a prerequisite for correct folding of the Gla domain.

MATERIALS AND METHODS

Preparation of subcellular particles

All solutions used for preparation of subcellular particles contained the following proteinase inhibitor mix: di-isopropyl fluorophosphate, 2.5 mM; benzamidine, 5 mM, leupeptin, 12.5 μg/ml; aprotinin, 12.5 μg/ml; *p*-amidinophenylmethanesulphonyl fluoride, 5 μg/ml; E-64, 5 μg/ml.

Microsomes and the Golgi apparatus were prepared from livers of 300 g male Sprague–Dawley rats as described in [4]. The subcellular particles were stored as pellets at −70 °C until used for the experiments.

Isolation of the prothrombin precursors from the Golgi apparatus

The Golgi apparatus particles were dissolved in 25 mM imidazole, pH 7.2, containing 0.5 M NaCl, 10% glycerol, 1.5% Triton X-114 and the inhibitor mix. This detergent extract of Golgi proteins was mixed with anti-(rat prothrombin) antibodies coupled to CNBr-activated Sepharose and the suspension was allowed to react overnight by shaking at 4 °C. The immunoaffinity resin was packed in a column and washed with 25 mM imidazole, 1% CHAPS and 10% glycerol, pH 7.2, containing 0.5 M NaCl and subsequently with the same buffer containing 2 M NaBr instead of NaCl. The prothrombin precursors were

Abbreviations used: Gla, γ-carboxyglutamic acid; PVDF, poly(vinylidene difluoride); PBS, 10 mM KH_2PO_4/150 mM NaCl, pH 7.2; E-64, *trans*-epoxysuccinyl-1-leucylamido-(4-guanidino)butane.

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eluted from the column in 0.1 M acetate, pH 4.0, containing 4 M urea, 0.5 M NaCl and 1% CHAPS. The precursors were precipitated from the urea-containing buffer by addition of 5 vol. of ice-cold acetone. Precipitation was carried out overnight at −20 °C. The precipitate was washed with cold 10% trichloroacetic acid and then with ether/ethanol (1:1, v/v).

Antibodies

Rabbit antisera raised against purified rat plasma prothrombin and the prothrombin propeptide were obtained as described [14]. Conformation-specific and conformation-independent antibodies to prothrombin were prepared from a 50% ammonium sulphate protein precipitate of antiserum. The precipitate was dissolved in PBS containing 5 mM benzamidine (buffer A) and dialysed extensively against this buffer before the IgG fraction was loaded on to an affinity column with purified rat plasma prothrombin coupled to CNBr-activated Sepharose. A fraction of conformation-specific antibodies (Ab2) was eluted from the column with buffer A containing 20 mM EDTA. The column was then washed with buffer A containing 1 M NaCl, before a fraction of conformation-independent anti-prothrombin antibodies (Ab1) was eluted with 0.1 M sodium acetate, pH 4.0, containing 4 M urea and 0.5 M NaCl. Both antibody fractions were dialysed against PBS containing 0.1% NaN_3 (buffer B) and concentrated in an Amicon cell on a PM-10 filter. A population of highly purified conformation-specific antibodies was obtained after re-chromatographing the Ab2 antibodies on the prothrombin affinity column in 50 mM Tris/HCl and 5 mM EDTA, pH 7.4. The void volume fraction of protein was collected and dialysed against buffer B. The two preparations of antibodies were stored at 4 °C.

SDS/PAGE and immunoblotting

One-dimensional SDS/PAGE was carried out in 10% gels according to Laemmli [15]. Two-dimensional SDS/PAGE was carried out by a modified version of the procedure of O'Farrell [16], as described in [17]. One-dimensional and two-dimensional immunoblots of the SDS/PAGE gels were obtained after transferring the electrophoresed proteins on to poly(vinylidene difluoride) (PVDF) membranes as described [4]. The second antibodies used for detection of immunoreactive proteins were either horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-rabbit IgG. The second antibody used in the various experiments is specified in the figure legends.

Immunodot-blotting

Recognition of purified rat plasma prothrombin by conformation-specific and -independent antibodies, in the presence or absence of EDTA, was tested in a blotting system using BSA as the membrane-blocking protein. The blotting procedure was carried out as described [14], except that the blotting buffer contained 5% BSA and either 10 mM EDTA or 10 mM Ca^{2+}. Antibody binding was estimated by reacting the membranes with 0.1 μCi/ml ^{125}I-Protein A and subjecting the immunoreactive spots to scintillation counting. Purified plasma prothrombin was used as standard.

Endoglycosidase digestions

A 25 μl sample of purified rat plasma prothrombin in PBS (30 μg/ml) was mixed with 1.3 μl of 10% SDS in water and 1.0 μl of mercaptoethanol. The mixture was boiled for 2 min, whereupon 26 μl of 7.5% Triton X-100 in water and 1.5 μl of N-Glycanase stock (0.4 unit) were added. The complete mixture was incubated at 37 °C overnight. The reaction was stopped by addition of 2 ml of ice-cold acetone. The protein precipitate was dissolved by boiling for 2 min in the SDS/PAGE electrophoresis buffer [15] containing 2% SDS and 5% β-mercaptoethanol.

Densitometric scanning of membranes

Scanning and integration of alkaline phosphatase-stained immunoreactive areas on the PVDF membranes were performed with an LKB Ultroscan XL Laser Densitometer which was programmed to read the scanned area 10 times to give an average of the integrated area. All measurements were made in the linear range of absorption.

Materials

PVDF membranes (Immobilon P) were from Millipore, Bedford, MA, U.S.A. Horseradish peroxidase- and alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies were from Bio-Rad, Richmond, CA, U.S.A. Purified rat plasma prothrombin and anti-(rat prothrombin) IgG were coupled to CNBr-activated Sepharose as recommended by the supplier (Pharmacia Fine Chemicals, Uppsala, Sweden). Prior to coupling, IgG was purified on Affigel–Protein A purchased from Bio-Rad. Recombinant N-Glycanase was from Genzyme, Boston, MA, U.S.A.

RESULTS

Two populations of antibodies raised against plasma prothrombin were used for these studies. The Ab1 antibodies, which we call the 'independent' antibodies, did not recognize epitopes defined by the Gla domain in prothrombin. On the other hand, the Ab2 antibodies were directed against the Ca^{2+}-induced Gla domain, and thus were conformation-specific for the domain. The specificities of the two different populations of antibodies are shown in Table 1. The independent antibodies (Ab1) did not discriminate between prothrombin molecules exposed to either 10 mM Ca^{2+} or 10 mM EDTA. On the other hand, the conformation-specific antibodies (Ab2) had no detectable affinity for prothrombin in the presence of EDTA, but clearly recognized prothrombin in the presence of Ca^{2+}. Varying the Ab1 and the Ab2 antibody concentrations did not change the pattern of antibody recognition shown in Table 1. These data demonstrate that the Ab2 antibodies could be used for identification of the correctly folded Gla domain in prothrombin.

The pool of prothrombin precursors located in microsomal vesicles, derived from the endoplasmic reticulum, were poorly

Table 1 Recognition of plasma prothrombin by conformation-specific and -independent antibodies

Two different pools of antibodies (Ab1 and Ab2) raised against rat plasma prothrombin were isolated from whole antiserum by affinity chromatography on prothrombin–Sepharose, as described in the Materials and methods section. The ability of Ab1 and Ab2 to recognize purified plasma prothrombin in the presence of 10 mM Ca^{2+} or 10 mM EDTA was tested by immunodot-blotting. The experiment was carried out with 30 ng of purified prothrombin attached to the PVDF membrane. The protein concentrations of Ab1 and Ab2 antibodies in the blotting buffer were 2.8 and 1.6 μg/ml respectively. Antibody recognition was estimated with ^{125}I-Protein A. The results are the means ± S.D. of four measurements.

	10^{-2} × Antibody recognition (c.p.m.)	
Addition to test system	Ab1	Ab2
Ca^{2+}	81 ± 3	65 ± 2
EDTA	84 ± 3	~ 0

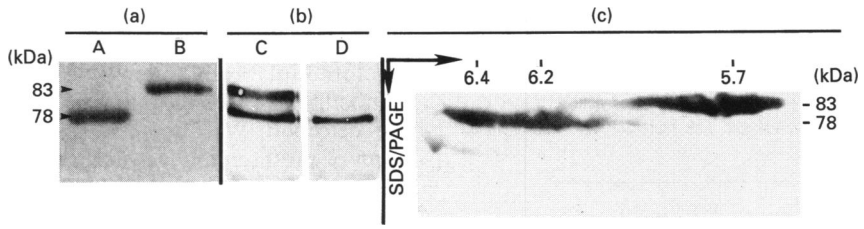

Figure 1 Immunoblotting of prothrombin and its intracellular precursors

(**a**) Immunoblots of the microsomal prothrombin precursors (lane A; 1.2 μg) and purified plasma prothrombin (lane B; 1.0 μg). (**b**) Lane C shows an immunoblot of the prothrombin precursors isolated from the Golgi apparatus when the blot was developed with the independent Ab1 antibodies. Lane D contains the same Golgi prothrombin precursors as lane C, but this immunoblot was developed with an antiserum directed against the prothrombin propeptide [4]. (**c**) Two-dimensional immunoblots of the prothrombin precursors isolated from the Golgi apparatus; numbers across the top indicate pH values. All blots, except the blot shown in lane C of (**b**), were developed with the independent Ab1 antibodies. The second antibody was horseradish peroxidase-conjugated IgG. Apparent molecular masses of the immunoreactive bands are indicated.

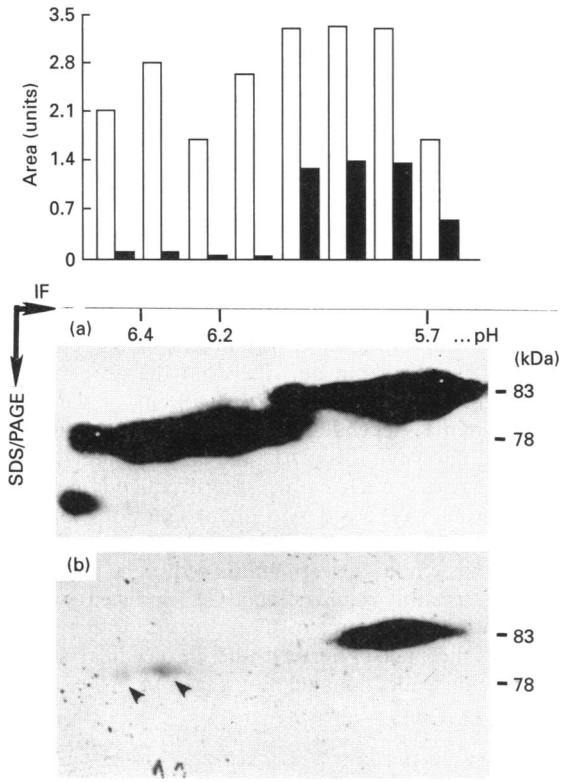

Figure 2 Immunoblotting of the Golgi apparatus prothrombin precursors with conformation-specific (Ab2) and -independent (Ab1) antibodies

Panels (**a**) and (**b**) show representative two-dimensional immunoblots of the prothrombin precursors isolated from the Golgi apparatus when the blots were developed with the independent (Ab1) and the conformation-specific (Ab2) antibodies respectively. The second antibody was alkaline phosphatase-conjugated IgG. The bar graphs show the average scanned areas of selected parts of the immunoblots. The positions of the bars correspond to the areas selected for scanning. Open bars correspond to panel (**a**), and filled bars correspond to panel (**b**). Exactly the same quantity of precursors (5.0 μg/ml) was present on both blots (**a**) and (**b**). The protein concentrations of Ab1 and Ab2 antibodies in the blotting buffer, which also contained 10 mM Ca^{2+}, were 2.8 μg/ml and 1.6 μg/ml respectively. IF, isoelectric focusing.

recognized by the conformation-specific Ab2 antibodies. These precursors that are present in early processing in the secretory pathway are a mixture of prothrombin precursors in various stages of γ-carboxylation, but all precursors have the propeptide attached [4]. Consistent with results obtained by Swanson and Suttie [18], the microsomal prothrombin precursor pool was shown to bind < 10% of the Ab2 antibodies per μg of precursor when compared with their binding to plasma prothrombin (100%). Thus the microsomal prothrombin precursors and plasma prothrombin could be regarded as the start and end points respectively of a series of intracellular events along the secretory pathway leading to mature prothrombin having correct Ca^{2+}-induced folding of the Gla domain.

Figure 1(a) shows immunoblots of the 78 kDa microsomal prothrombin precursors (lane A) and plasma prothrombin (83 kDa) (lane B). Figure 1(b) shows immunoblots of the prothrombin precursors isolated from the Golgi apparatus, the next set of intracellular organelles along the secretory pathway participating in prothrombin processing [4]. Consistent with previous results [4], the immunoblot in Figure 1(b), lane C, demonstrates that the Golgi apparatus prothrombin precursors, after being subjected to SDS/PAGE, appear as two pools of proteins with apparent molecular masses of 78 and 83 kDa. Lane C was developed with the Ab1 independent antibodies. Lane D in Figure 1(b) demonstrates that only the 78 kDa precursors are recognized by an antiserum raised against the prothrombin propeptide. Previously we have demonstrated that the 78 kDa prothrombin precursors are derived from the cis- and medial-Golgi compartments and that the propeptide is associated with the prothrombin precursor in both compartments [4]. The 83 kDa Golgi prothrombin precursor, which is devoid of the propeptide, is derived from the trans-Golgi apparatus. This conclusion is based on the findings that: (1) these precursors have the same molecular mass as plasma prothrombin; (2) they are insensitive to endonuclease H [4]; (3) they are the most acidic of the intracellular prothrombin precursors, as shown by two-dimensional immunoblotting in Figure 1(c); and (4) the propeptide-releasing endoproteinase furin is located in the trans-Golgi apparatus. Thus two-dimensional immunoblotting of the Golgi apparatus prothrombin precursors provided us with a test system which could be used to decide whether or not release of the propeptide in the trans-Golgi apparatus is essential for Ca^{2+}-induced folding of the Gla domain.

Figure 2(a) shows a two-dimensional immunoblot of the Golgi apparatus prothrombin precursors when the blot was developed with the independent Ab1 antibodies. Figure 2(b) shows this two-dimensional immunoblot developed with the conformation-specific Ab2 antibodies. The bar graph shows the integrated areas of selected parts of the blots, and the positions of the bars correspond to the areas selected. The open bars, which correspond to Figure 2(a), provide estimates of the densities of the prothrombin precursors at various sites in the Golgi apparatus. The solid bars provide estimates of these density values when the

Table 2 Quantitative antibody recognition of glycosylated and deglycosylated prothrombin

The immunoblots shown in Figures 3(a) and 3(b), were each scanned with a laser densitometer at four different locations to obtain average densities which were representative for each band. Integrated areas (means ± S.D.) are shown. Ab1 and Ab2 represent the independent and conformation-specific antibodies respectively.

	Density (units)	
Protein	Ab1	Ab2
Prothrombin (lanes A and C, Figure 3)	2.9 ± 0.2	1.9 ± 0.1
Deglycosylated prothrombin (lanes B and D, Figure 3)	2.7 ± 0.2	1.8 ± 0.1

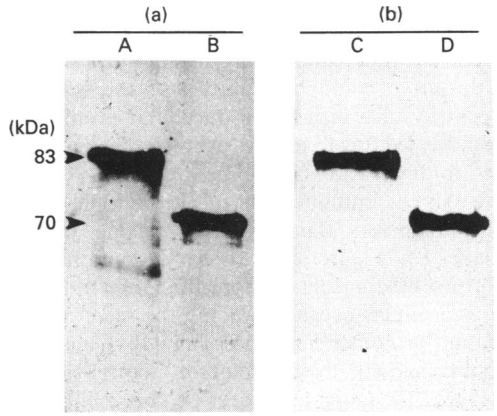

Figure 3 Antibody recognition of deglycosylated prothrombin

Immunoblots of purified prothrombin developed with (**a**) the independent (Ab1) and (**b**) the conformation-specific (Ab2) antibodies. The Ab1 and Ab2 antibody concentrations in the blotting buffer were as described in the legend to Table 1. Lanes B and D show prothrombin after N-Glycanase treatment; lanes A and C show prothrombin in control incubations without N-Glycanase. Lanes contained the same amount of prothrombin (0.6 μg).

conformation-specific antibodies (Ab2) were used for detection. This experiment was repeated with three different preparations of prothrombin precursors from the Golgi apparatus. The mean ratio between the scanned areas in Figures 2(a) and 2(b), as defined by the Ab1 and Ab2 antibodies, (area Ab1/area Ab2), decreased from 37 ± 2 ($n = 3$) to 2.5 ± 0.3 ($n = 3$) when going from the cis/medial-Golgi compartment (arrowheads in Figure 2b) to the trans-Golgi compartment (83 kDa proteins). A ratio of 2.5 was also measured when the blotting procedure was carried out under identical conditions with purified plasma prothrombin (results not shown). These data demonstrate that full recognition of the Gla domain in prothrombin was not achieved before the precursors entered the more acidic trans-Golgi compartment of the secretory pathway.

Figure 3 and Table 2 present evidence that the complex Asn-linked carbohydrate moieties on prothrombin were not part of the epitope that determines the specificity of the conformation-specific Ab2 antibodies. Figures 3(a) and 3(b) show recognition of plasma prothrombin by Ab1 and Ab2 respectively, before (lanes A and C) and after (lanes B and D) N-Glycanase treatment of the protein. N-Glycanase converted the 83 kDa prothrombin molecule to its deglycosylated 70 kDa form [4]. However, as shown in Table 2, densitometric scanning of the immunoblots in Figure 3 did not detect any difference in antibody recognition of the glycosylated (lanes A and C) and the deglycosylated (lanes B and D) forms of prothrombin whether the conformation-specific antibodies (Ab2) or the independent antibodies (Ab1) were used for detection. We also demonstrated that the deglycosylated form of prothrombin was not recognized by the conformation-specific antibodies in the presence of 5 mM EDTA (results not shown).

DISCUSSION

This paper provides evidence that the propeptide, when attached to the N-terminus of prothrombin, prevents the Ca^{2+}-induced folding of the Gla domain which is necessary for prothrombin to be normally activated in the coagulation system. The conformational change is a required event in formation of the prothrombinase complex and activation of prothrombin to thrombin [6]. Our data are also in accordance with those of Welsch and Nelsestuen [19], who showed that chemical acetylation of the N-terminal Ala in plasma prothrombin prevents folding of the Gla domain, indicating that a free N-terminus is important for folding. Our data suggest that the propeptide, when attached to otherwise fully γ-carboxylated proteins, will prevent folding of the Gla domain and result in bleeding disorders. This finding indicates a very important role for furin, the trans-Golgi endoproteinase involved in propeptide release [4] in maintaining a normal haemostatic system. This idea is supported by the fact that furin has also been shown to be involved in proteolytic processing of the von Willebrand factor [20].

Another interesting observation also emerged from these studies. It is clear from our data that the propeptide exerts a natural constraint on Ca^{2+}-induced folding of the Gla domain when vitamin K-dependent proteins undergo intracellular processing. As shown in this work, and also in a previous paper [4], propeptide release is a late processing event and therefore follows completion of γ-carboxylation of the Gla domain, which is an early processing event [4]. The endoplasmic reticulum and probably also the Golgi apparatus sequester large Ca^{2+} stores [21], which certainly would favour a Ca^{2+}-induced conformational change in the Gla domain. The propeptide may represent a protective mechanism against the intracellular activation of vitamin K-dependent coagulation factors.

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