Biochem. J. (1991) 277, 59–65 (Printed in Great Britain)

Processing of prothrombin in the secretory pathway

Catherine STANTON, Richard TAYLOR and Reidar WALLIN*
Department of Medicine, Section on Rheumatology, Bowman Gray School of Medicine, Wake Forest University, 300 South Hawthorne Road, Winston-Salem, NC 27103, U.S.A.

Antibodies raised against plasma prothrombin and the prothrombin propeptide were used to identify prothrombin precursors in rough and smooth microsomes and in the Golgi apparatus. The data demonstrate that the propeptide is part of the prothrombin molecule when undergoing a variety of modifications in the Golgi apparatus. It is shown that these modifications result in an increase in the apparent molecular mass of the prothrombin precursor from 78 kDa in early processing to 83 kDa in late processing. The 83 kDa prothrombin precursor was not recognized by the anti-propeptide antiserum and most likely represents the final product of the precursor in the secretory pathway. Evidence is presented that the propeptide is released from the parent molecule in the Golgi apparatus by a membrane-bound Ca^{2+}-dependent serine proteinase(s) with characteristics similar to those of the proalbumin-to-albumin-converting enzyme. Vitamin K-dependent carboxylase activity was measured in membrane fragments obtained from the Golgi apparatus preparation. Sucrose-density-gradient centrifugation and the use of marker enzymes showed that carboxylase activity was highest in fractions enriched in cis-Golgi cisternae. Two different synthetic peptides were used as substrates for the carboxylase. These peptides were from the *N*-terminal and the *C*-terminal part of the γ-carboxyglutamic acid (Gla) region of prothrombin. It is shown that the *N*-terminal and the *C*-terminal peptides were preferred as substrates for the carboxylase in the microsomal and the Golgi apparatus preparations respectively. It is also shown that the prothrombin precursor acquires negative charges in the Golgi apparatus that do not result from addition of sugars in late processing. These negative charges could be eliminated by thermal decarboxylation, suggesting that Gla residues may also be synthesized in late processing.

INTRODUCTION

Vitamin K-dependent clotting factors are high-mannose glycoproteins that are synthesized in the liver [1]. Prior to secretion of these proteins into plasma as zymogens of the coagulation system, they undergo extensive postribosomal modifications. These modifications include vitamin K-dependent γ-carboxylation of specific glutamic acid residues in the polypeptide chain, converting them to γ-carboxyglutamic acid (Gla) residues [2]. The finished plasma proteins have 10–12 Gla residues residing in the *N*-terminal part of the proteins [3]. Formation of Gla residues in the newly synthesized proteins is believed to be confined to the endoplasmic reticulum as an early processing event catalysed by the vitamin K-dependent carboxylase [2]. The carboxylase is an integral membrane protein of the endoplasmic reticulum which requires reduced vitamin K_1H_2, CO_2, and O_2 for the γ-carboxylation reaction [4].

The vitamin K-dependent clotting factor carboxylase substrates are synthesized with a propeptide that is positioned in the precursor protein sequence between the signal peptide and the *N*-terminus of the mature protein [2]. Several lines of evidence suggest that the propeptide is the site recognized by the carboxylase for sorting of these proteins for γ-carboxylation [2,5,6]. In addition, the propeptide has been shown to modulate carboxylase activity, which suggests that there is also a regulatory role for the propeptide in processing of vitamin K-dependent proteins [7]. The propeptide is released from the parent molecule before the coagulation zymogen appears in plasma.

The aim of the present work was to determine the site of prothrombin propeptide cleavage in the secretory pathway and to identify the endoproteinase(s) involved. The data provide conclusive evidence for propeptide-containing prothrombin precursors in the Golgi apparatus and identifies the endoproteinase as a Ca^{2+}-dependent serine proteinase. Finally, we present data which suggests that γ-carboxylation may also be a late processing event.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (250–300 g; Zivic Miller Laboratories Inc., Zelienople, PA, U.S.A.) were used for the experiments. For preparation of subcellular particles, the animals were first anaesthesized with pentobarbital and the livers perfused through the portal vein with ice-cold 0.9% NaCl.

Preparation of microsomal particles

Liver microsomes from perfused livers were prepared as described previously [8]. Separation of liver microsomes into rough and smooth was carried out as described by Dallner [9] using a sucrose/CsCl gradient.

Preparation of particles from the Golgi apparatus

The procedure used for preparation of these particles was a combination of the methods described by Croze & Morré [10] and Fleischer [11]. All steps were carried out at 4 °C. About 300 g of perfused liver tissue was minced and homogenized in 2 vol. of 37.5 mM-Tris/maleate buffer, pH 7.0, containing 1% dextran (average molecular mass 298 kDa), 0.5 M-sucrose, 5.0 mM-$MgCl_2$, 0.1% 2-mercaptoethanol, 5 mM-benzamidine and 1 mM-di-isopropyl fluorophosphate (DFP) (buffer A). Homogenization was carried out for 1 min in a Tekmar Polytron at 35% of maximum setting. The homogenate was centrifuged for 10 min at 8000 *g* in a fixed-angle JA-18 rotor (Beckman Instruments) to give pellet I and a supernatant. The supernatant was subjected to a second centrifugation at 10000 *g* in the same rotor to obtain a

Abbreviations used: *p*-APMSF, *p*-amidinophenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; E-64, *trans*-epoxysuccinyl-l-leucylamido-(4-guanidino)butane; DFP, di-isopropyl fluorophosphate; Gla, carboxyglutamic acid; endo H, endoglycosidase H.

* To whom correspondence and reprint requests should be sent.

pellet (pellet II) highly enriched in Golgi particles. To increase the yield of Golgi particles in the final preparation, the upper layer of pellet I (one-quarter total pellet) was removed and resuspended in buffer A. The resuspended material was then centrifuged at 8000 *g* for 10 min in a JS-13.1 Beckman swing-out rotor. The upper, light yellow, layer of the pellet was removed, combined with pellet II and resuspended in buffer A. Portions (5 ml) of the suspension were layered over 8 ml of 1.2 M-sucrose in 37.5 mM-Tris/maleate buffer, pH 7.0, containing 5 mM-benzamidine and 1 mM-DFP and centrifuged for 30 min at 100000 *g* (28000 rev./min) in an SW 40 rotor. The material appearing at the interface was collected and suspended in 25 mM-imidazole/HCl buffer, pH 7.2, containing 250 mM-sucrose, 5 mM-benzamidine and 1 mM-DFP (SI buffer). The material was pelleted by centrifugation at 10000 *g* for 10 min in the JA-18 rotor. The pellet was resuspended in 43.7% sucrose, and 2 ml samples were overlayed with 3 ml of 38.7% sucrose, 3 ml of 36% sucrose, 2 ml of 33% sucrose and 3 ml of 28% sucrose in 14 mm × 95 mm POLYallomer tubes. They were spun for 60 min at 100000 *g* (28000 rev./min) in an SW 40 rotor. For analysis of the gradient, 10 fractions were collected by slow aspiration from the top to the bottom. Each fraction was diluted with phosphate-buffered saline (0.01 M-KH_2PO_4/0.15 M-NaCl, pH 7.2) and pelleted by centrifugation at 100000 *g* for 45 min. The pelleted material was resuspended in the appropriate buffer used for the various enzyme assays. On a routine basis, the material sedimenting at the 33%/28% interface was collected as the final Golgi apparatus preparation. The Golgi apparatus preparation was diluted in SI buffer and pelleted by centrifugation at 100000 *g*. The pellets were stored at −70 °C.

Preparation of membrane fragments

Pellets of isolated subcellular particles were suspended in 25 mM-imidazole/HCl, pH 7.2, containing 0.5% CHAPS, 5 mM-benzamidine and 1 mM-DFP and left shaking on ice for 30 min. The membrane fragments that were insoluble in the detergent-containing buffer were isolated by centrifugation at 100000 *g* for 45 min. The pelleted membrane fragments were surface-washed once in the centrifuge tube with the detergent-containing buffer before use.

Electron microscopy

Ultrastructural examination was carried out to assess the purity of isolated subcellular particles. Fractions of rough and smooth microsomes and Golgi particles were fixed using 1% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.3. After 1 h of fixation, the samples were rinsed with cacodylate buffer, post-fixed in 1% osmium in cacodylate buffer, then dehydrated in a graded series of ethanol and embedded in Spurr's resin. These sections were stained with lead citrate and uranyl acetate before observation at 80 kV in a Philips EM-400 microscope.

Enzyme assays

CMP-NeuAc:glycoprotein sialyltransferase (EC 2.4.99.1) activity was measured by a modification of the method described by Berger & Hesford [12] in 0.1 ml reaction mixtures containing 0.5 mM-CMP-[G-^{3}H]NeuAc (20 μCi/μmol) and 0.65 mg of the acceptor protein asialofetuin. Incubations were carried out for 30 min at 37 °C.

GlcNAc-1-phosphotransferase (EC 2.7.8.17) was assayed as described by Reitman & Kornfeld [13] using methyl α-mannoside as acceptor and UDP-*N*-acetyl[6-^{3}H]glucosamine as donor [14].

Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to Noordlie & Arion [15].

Vitamin K-dependent carboxylase activity was determined in membrane fragments prepared from subcellular particles. Incubations contained 1 mM-$MnCl_2$, 5 mM-dithiothreitol, 4 mM of a synthetic peptide carboxylase substrate, 20 μCi of $NaH^{14}CO_3$/ml and 100 μg of chemically reduced vitamin K_1H_2/ml. Activity was determined as $^{14}CO_2$ incorporation into the synthetic peptide, and the assay was carried out as described [16].

Peptide synthesis

The propeptide His-Val-Phe-Leu-Ala-Pro-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg-Ala from the human prothrombin sequence was synthesized by Bachem Fine Chemicals, Torrence, CA, U.S.A. The peptide was guaranteed by the supplier to be > 99% pure, based on h.p.l.c. analyses in three different chromatographic systems.

The peptides Ala-Asn-Thr-Phe-Leu-Glu-Glu-Val-Arg (peptide I) and Ala-Phe-Glu-Ala-Leu-Glu-Ser-Ser-Ala (peptide II) were synthesized by the Protein Core Laboratory at The Bowman Gray School of Medicine, Winston-Salem, and were > 98% pure. These peptides represented the *N*-terminal and *C*-terminal parts of the Gla region in human prothrombin respectively. Peptide I contained the first two Glu residues, whereas peptide II contained and the last two Glu residues of the Gla region respectively [17].

Antisera

An antiserum against the synthetic prothrombin propeptide was raised in rabbits (female, New Zealand White, 10 weeks old), as described by Atassi [18]. The peptide (500 μg) was emulsified in Freund's complete adjuvant and given intradermally at multiple sites over the entire back of the animal. After 4 weeks the rabbits were boosted with 500 μg of the peptide in Freund's incomplete adjuvant.

Blood was drawn 1 week after the last injection. Serum was stored at −20 °C. Preimmune serum was obtained by drawing 10 ml of blood from the rabbits before immunization. Rabbit antiserum against rat plasma prothrombin was obtained as described [16].

Endoglycosidase H (endo H) and *N*-Glycanase treatment of glycoproteins

Protein samples were digested with endo H (EC 3.2.1.30) as described previously [16]. The samples were treated with 10 units of endo H at 37 °C overnight in buffer containing 0.3% SDS and 1% 2-mercaptoethanol. *N*-Glycanase (EC 3.2.2.18) treatment of protein was performed on samples denatured by boiling for 2 min in the presence of 1% SDS and 1% 2-mercaptoethanol. To a 100 μl boiled sample was added 900 μl of 0.1 M-sodium phosphate, pH 7.8, containing 10 mM-EDTA, 0.6% Triton X-100 and 1.5 units of recombinant *N*-Glycanase. The protein sample was incubated with the enzyme at 37 °C overnight. Protein was precipitated with 10% trichloroacetic acid and pelleted in an Eppendorf tube. Trichloroacetic acid was removed with ice-cold diethyl ether/ethanol (1:1, v/v) before the sample was dissolved in the 9 M-urea-containing sample buffer used for isoelectric focusing (see below).

Immunoblotting

From one-dimensional SDS/PAGE gels. SDS/PAGE was carried out according to Laemmli [19] in 10% (w/v) gels. Before electrophoresis, all samples were boiled for 2 min in the presence of 5% 2-mercaptoethanol and 2% SDS. Standard proteins used for molecular mass determinations were prestained proteins purchased from Bio-Rad, Richmond, CA, U.S.A. Proteins were transferred to Immobilon P membranes (Millipore Corp., Bedford, MA, U.S.A.) in a Bio-Rad Trans-Blot Cell, and treated with antisera as described [16]. Immunoreactive proteins attached

to the Immobilon P membrane were visualized after horseradish peroxidase reduction of 4-chloro-1-naphthol [16].

From two-dimensional SDS/PAGE gels. Two-dimensional SDS/PAGE was carried out according to a modification of the O'Farrell method [20] as described in [21]. SDS/PAGE in the second dimension was carried out in 10% gels. The SDS/PAGE gel was rinsed for 10 min in water before proteins were transferred to an Immobilon P membrane as described above for one-dimensional PAGE. Immunoreactive proteins were visualized as described above.

Immunoadsorption

Protein samples used for immunoadsorption were solubilized in 25 mM-imidazole/HCl, pH 7.2, buffer containing 0.5 M-KCl, 1.5% Triton X-100, 250 mM-sucrose, 5 mM-benzamidine and 1 mM-DFP. In most experiments, 25 μl of antiserum was added to 1 ml of the solubilized sample and immunocomplexes were allowed to form overnight at 4 °C. Immunocomplexes were isolated either with *Staphylococcus aureus* Protein A particles as described in [16], or with Protein A attached to Affigel 702 particles purchased from Bio-Rad. Immunocomplexes were released from Protein A in the urea/Triton X-100 sample buffer used for isoelectric focusing.

Proteolytic release of the prothrombin propeptide

Membrane fragments from the isolated Golgi apparatus were suspended in 50 mM-Tris/HCl, pH 6.0, containing 5 mM-$CaCl_2$ and 1% Triton X-100 [22] and incubated for 2 h at 37 °C in the presence of various proteinase inhibitors and EGTA as described in the legend to Fig. 8. Protein in incubated samples was precipitated with 10% trichloroacetic acid and prepared for SDS/PAGE and immunoblotting as described above.

Thermal decarboxylation

Thermal decarboxylation of Gla residues was carried out as described by Poster & Price [23]. *N*-Glycanase-treated protein samples were lyophilized in 0.05 M-HCl and heated under a vacuum at 110 °C for 6 h.

Materials

Vitamin K_1, leupeptin, E-64, cystatin, aprotinin, *p*-amidinophenylmethanesulphonyl fluoride (*p*-APMSF), benzamidine, DFP and *N*-ethylmaleimide were purchased from Sigma. The vitamin was reduced to vitamin K_1H_2 as described by Sadowski *et al.* [24]. The carboxylase peptide substrate Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals, Tucson, AZ, U.S.A. $NaH^{14}CO_3$ (60 mCi/mmol) and endo H were from ICN Biochemicals, Costa Mesa, CA, U.S.A. Recombinant *N*-Glycanase was purchased from Genzyme, Boston, MA, U.S.A. *Staphylococcus aureus* Protein A (Pansorbin) and purified Protein A were from Calbiochem, San Diego, CA, U.S.A. Affigel 702 polyacrylamide beads were from Bio-Rad, and were used for immobilization of Protein A as recommended by the supplier. Prothrombin was purified from citrated rat plasma as described by Novoa *et al.* [25]. All reagents used for immunoblotting were from Bio-Rad. Protein was measured with the Bio-Rad protein assay.

RESULTS

Marker enzyme analysis of fractions from the sucrose density gradient used as the final step in purification of the Golgi apparatus is shown in Fig. 1. Fraction 2, which contained the particles sedimenting at the 33%/28% sucrose interface, had the most sialic acid transferase activity, a marker enzyme activity for the trans-Golgi cisternae [26]. GlcNAc-1-phosphotransferase activity, a marker enzyme activity for the cis-Golgi cisternae [27], was highest in fraction 4 (Fig. 1). The distribution of these two marker enzymes demonstrated that some separation of the cis- and trans-Golgi compartments had been achieved in fractions 2 and 4. Also shown in Fig. 1 is the distribution of glucose-6-phosphatase activity over the gradient. This marker enzyme activity for the endoplasmic reticulum [10] peaked at the bottom of the gradient (fraction 10). Glucose-6-phosphatase activity recovered in fractions 2 and 3 was 3% and 2% respectively of the total activity measured in the gradient.

As shown in Fig. 2, immunoblotting with anti-(plasma prothrombin) antiserum identified prothrombin antigens in rough (lane A) and smooth (lane B) microsomes and in the Golgi apparatus (lane C). Consistent with previous results [16], the microsomal prothrombin precursor appeared with a molecular mass of 78 kDa (lanes A and B). Three immunoreactive bands appeared on the immunoblot of the Golgi apparatus (lane C). The band with the apparent highest molecular mass co-migrated in the SDS/PAGE gel with plasma prothrombin (83 kDa, lane D). The more diffuse band in the middle co-migrated with the 78 kDa microsomal precursor. In addition, the Golgi apparatus revealed a heavily stained band of apparent molecular mass 60–65 kDa (Fig. 2, lane C). This band did not appear on immunoblots of the Golgi apparatus after these particles had

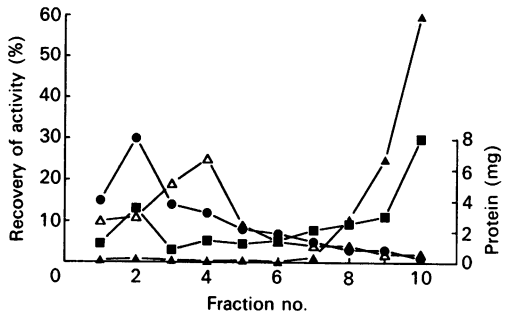

Fig. 1. Distribution of marker enzymes in the sucrose gradient used for isolation of the Golgi apparatus

Enzyme activities measured in the various fractions are presented as recovered activity as a percentage of total activity measured in the gradient (fractions 1–10). Fractions 1 and 10 represent the top and the bottom of the gradient respectively. For construction of the gradient, see the Materials and methods section. ▲, Glucose-6-phosphatase; △, GlcNAc-1-phosphotransferase; ●, CMP-NeuAc:glycoprotein sialyltransferase; ■, protein.

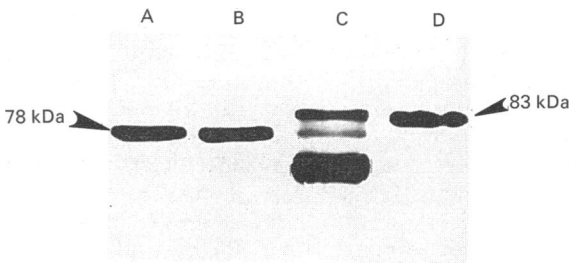

Fig. 2. Immunoblotting of subcellular particles and plasma with anti-prothrombin antiserum

Lanes A, B, C and D show immunoblots of rough and smooth microsomes, the Golgi apparatus and rat plasma respectively with rat anti-prothrombin antiserum. Antiserum was added to Blotto (see the Materials and methods section) in a ratio of 1:750. Molecular masses in kDa are shown for the microsomal prothrombin precursor and plasma prothrombin.

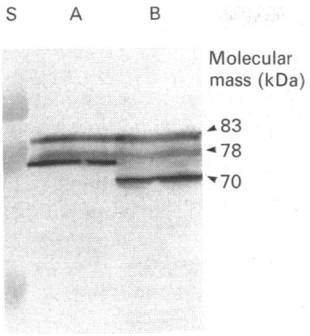

Fig. 3. Immunoblotting of Golgi apparatus membrane fragments with prothrombin antiserum

Golgi apparatus membrane fragments prepared as described in the Materials and methods section were immunoblotted with anti-prothrombin antiserum. Lanes A and B show immunoblots of the membrane fragments respectively before and after treatment of the fragments with endo H. Molecular masses in kDa for the various immunoreactive bands appearing on the blot are shown. Lane S contains pre-stained standard proteins.

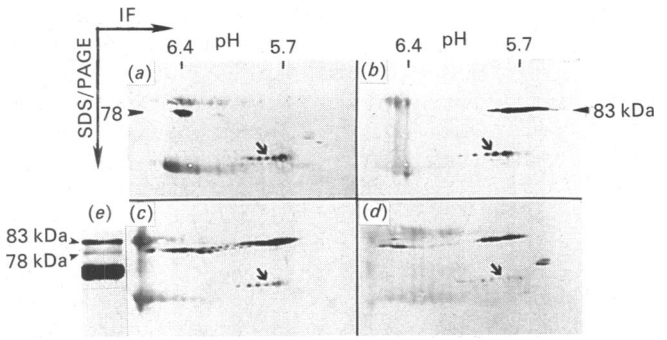

Fig. 4. Two-dimensional immunoblotting of prothrombin precursors and plasma prothrombin

Prothrombin antigens in microsomes (*a*), the Golgi apparatus (*c* and *d*) and rat plasma (*b*) were isolated as immunocomplexes with anti-prothrombin antibodies and subjected to two-dimensional SDS/PAGE and immunoblotting as described in the Materials and methods section. The immunoblots shown in (*a*), (*b*) and (*c*) were developed with anti-prothrombin antiserum. The immunoblot of the Golgi prothrombin precursors shown in (*d*) was developed with an antiserum raised against the prothrombin propeptide. Panel (*e*) shows a one-dimensional immunoblot of the prothrombin precursors in the Golgi apparatus. Molecular masses in kDa are indicated. IF, isoelectric focusing.

been washed with buffer containing a 0.5% concentration of the detergent CHAPS (Fig. 3). The band was apparently an artifact of analysis because immunocomplexes with prothrombin antibodies, when analysed by two dimensional immunoblotting, also did not show the 60–65 kDa protein (Figs. 4*c* and 4*e*).

A close inspection of the immunoblot of detergent-washed Golgi membranes (Fig. 3, lane A) showed that the 78 kDa band was composed of two protein bands of slightly different molecular mass. As shown in Fig. 3, lane B, the lower of these bands was sensitive to endo H digestion, which changed its apparent molecular mass to 70 kDa. The other 78 kDa band and also the 83 kDa band were insensitive to endo H (Figs. 3, compare lanes A and B).

Antibodies raised against plasma prothrombin and the synthetic prothrombin propeptide were used to investigate prothrombin precursors in the microsomal and Golgi apparatus preparations. Before analysis, the precursors were isolated as immunocomplexes with plasma anti-prothrombin antibodies. The immunocomplexes were subjected to two-dimensional SDS/PAGE and analysed by immunoblotting. Figs. 4(*a*), 4(*b*) and 4(*c*) show the microsomal prothrombin precursors, plasma prothrombin and the Golgi apparatus prothrombin precursors respectively when treated with prothrombin antibodies for immunoblotting. Fig. 4(*d*) shows the precursors in the Golgi apparatus after immunoblotting with the propeptide antiserum. Fig. 4 demonstrates a major shift in pI for the prothrombin precursors when going from early processing in the endoplasmic reticulum (Fig. 4*a*) via late processing in the Golgi apparatus (Fig. 4*c*) to become a mature plasma zymogen (Fig. 4*b*). The difference in pI between the most extreme forms was estimated to be 1 pH unit (Fig. 4*c*). The arrows shown on the different panels in Fig. 4 point to a set of proteins released from the *Staphylococcus aureus* particles simultaneously with release of the immunocomplexes when these particles were treated with 9 M-urea (see the Materials and methods section). This background staining could be used as a reference point for determining the relative migration of the prothrombin precursors in the direction of isoelectric focusing. This marker was used to measure migration of prothrombin precursors identified with the propeptide antiserum. As seen in Fig. 4(*d*), the propeptide antiserum identified prothrombin precursors with a wide range of pI values. However, the most acidic forms of the prothrombin precursor (pI > 5.7) that could be identified with anti-prothrombin antibodies were not recognized by the anti-propeptide antibodies (compare Figs. 4*c* and 4*d*). This experiment provided evidence that the propeptide is part of the prothrombin molecule when the latter is undergoing a variety of modifications in late processing. Also, the experiment demonstrated that the modifications that the prothrombin molecule undergo in late processing increase its apparent molecular mass from 78 kDa to 83 kDa (Figs. 4*c* and 4*e*).

The observation of the propeptide in prothrombin precursors in late processing raised questions about propeptide function in the Golgi apparatus. Since the propeptide has been shown to be the carboxylase recognition site, experiments were carried out to determine whether or not vitamin K-dependent carboxylation could be measured in our preparation of the Golgi apparatus. Two different nine-residue synthetic peptides were used as substrates for the carboxylase. Peptide I was the *N*-terminal part of human prothrombin harbouring the first two most *N*-terminal Glu residues, which appear as Gla residues in mature prothrombin [17]. Peptide II contained the final two most *C*-terminal Glu residues of the Gla region (see the Materials and methods section). Fig. 5 shows the distribution of carboxylase activity in fractions from the sucrose gradient used for determination of marker enzyme activities in Fig. 1. Carboxylase activity was present in all fractions from the gradient, with the most activity measured in fraction 10, the fraction shown in Fig. 1 to contain microsomal particles. Among the other fractions from the gradient, fraction 4, the cis-Golgi-enriched fraction (see Fig. 1), had the most carboxylase activity, suggesting that this activity may be associated with the cis-part of the Golgi apparatus. However, carboxylase activity measured with peptide I and peptide II respectively was distributed unevenly over the gradient. In fractions enriched in microsomal particles (fractions 7–10, Fig. 5) peptide I yielded higher activity than peptide II. On the other hand, in fractions enriched in the Golgi apparatus, peptide II resulted in the highest activity (fractions 1–6, Fig. 5). Specific carboxylase activity measured in the Golgi-apparatus-containing fractions was, however, significantly lower than specific carboxylase activity measured in liver microsomes. For fraction 4 from the sucrose gradient (see Fig. 5), these measurements were 31% and 65% of the microsomal carb-

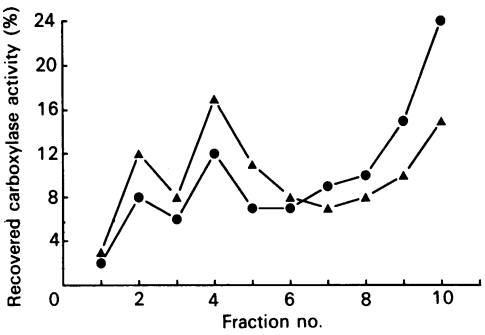

Fig. 5. Distribution of vitamin K-dependent carboxylase activity in the sucrose gradient used for isolation of the Golgi apparatus

Carboxylase activities measured in the various fractions are presented as a percentage of total activity measured in the gradient (fractions 1–10). For construction of the gradient, see the legend to Fig. 1 and the Materials and methods section. ●, Carboxylase activity measured with peptide I as substrate; ▲, carboxylase activity measured with peptide II as substrate.

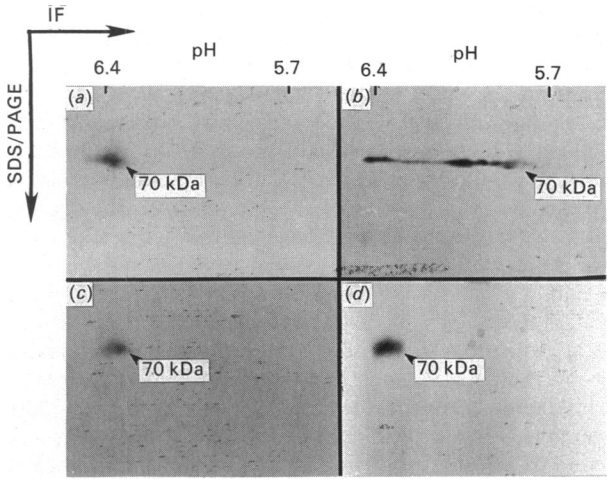

Fig. 6. Two-dimensional immunoblotting of *N*-Glycanase-treated and decarboxylated prothrombin precursors

Prothrombin antigens in microsomes (*a* and *c*) and the Golgi apparatus (*b* and *d*) were isolated as immunocomplexes with anti-prothrombin antibodies and subjected to *N*-Glycanase treatment and thermal decarboxylation as described in the Materials and methods section. (*a*) and (*b*) show the immunoblots of *N*-Glycanase-treated microsomal and Golgi prothrombin precursors respectively. Panels (*c*) and (*d*) show immunoblots of thermally decarboxylated and *N*-Glycanase-treated microsomal and prothrombin precursors respectively. Apparent molecular masses in kDa are indicated. IF, isoelectric focusing.

oxylase activity when peptides I and II respectively were used as substrates.

Additional data to support the notion of γ-carboxylation in late processing are presented in Fig. 6. In this experiment, isolated prothrombin precursors from microsomes and the Golgi apparatus were deglycosylated with *N*-Glycanase in order to evaluate the contribution of sugar residues to the negative charges acquired by the prothrombin precursors when going from early processing in the endoplasmic reticulum to late processing in the Golgi apparatus. Figs. 6(*a*) and 6(*b*) show two-dimensional immunoblots of the *N*-Glycanase-treated microsomal and Golgi precursors respectively. As shown (Fig. 6*a*), *N*-Glycanase had an insignificant effect on the pI values of the microsomal precursors, but changed their apparent molecular

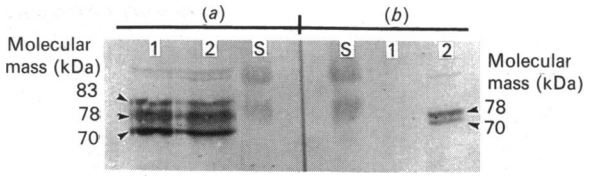

Fig. 7. Identification of prothrombin propeptide release in the Golgi apparatus

Golgi apparatus membrane fragments treated with endo H were used for the experiments. Treated membranes (*a* and *b*, lane 2) and treated membranes incubated for 2 h in a Ca^{2+}-containing buffer, pH 6.0 (*a* and *b*, lane 1) (see the Materials and methods section), were electrophoresed into a SDS/PAGE gel (10%) for immunoblotting. Panels (*a*) and (*b*) show immunoblots with anti-prothrombin and anti-(prothrombin propeptide) antisera respectively. Apparent molecular masses in kDa are indicated for immunoreactive bands. Lane S contains pre-stained standard proteins.

mass from 78 kDa to 70 kDa, consistent with the effect of endo H on these precursors [16]. The *N*-Glycanase-treated prothrombin precursors from the Golgi apparatus revealed a line of proteins on the two-dimensional immunoblot which had the same molecular mass (70 kDa) but with pI values ranging from 6.4 to 5.9 (Fig. 6*b*). This experiment demonstrated that modifications in the Golgi apparatus other than glycosylation were responsible for charge differences observed among various prothrombin precursors in the Golgi apparatus. The result shown in Fig. 6(*d*) suggests that these negative charge differences may have resulted from Gla residues acquired by the prothrombin precursors in late processing. As shown in Fig. 6(*d*), thermal decarboxylation of the *N*-Glycanase-treated prothrombin precursors, using a standard method for decarboxylation of Gla residues [23], resulted in a loss of negative charges and the appearance of prothrombin precursors with pI values of 6.4–6.5 on the two-dimensional immunoblot. On the other hand, thermal decarboxylation of *N*-Glycanase-treated microsomal prothrombin precursors had very little effect on their pI (Fig. 6*c*).

As shown in Fig. 7(*b*), lane 2, the anti-propeptide antiserum did not recognize the 83 kDa prothrombin precursor found in the isolated Golgi apparatus. In order to demonstrate release of the propeptide by proteolysis in the Golgi apparatus, we incubated washed Golgi membranes under conditions known to be optimal for proteolytic processing of proalbumin to albumin [28] and subsequently tested for the presence of the propeptide. In this experiment the Golgi membranes had been pretreated with endo H, which made the 'early' prothrombin precursors appear as the 70 kDa band on the immunoblot shown in Fig. 7. As seen in Fig. 7(*b*), lane 2, the anti-propeptide antiserum recognized the propeptide in both the 78 and the 70 kDa proteins from the control sample. No immunoreaction with this antiserum could be seen, however, after the sample had been incubated as described above (Fig. 7*b*, lane 1). On the other hand, the anti-prothrombin antiserum recognized proteins with the same apparent molecular mass both in the control sample (Fig. 7*a*, lane 2) and in the incubated sample (Fig. 7*a*, lane 1). The experiment demonstrated that: (1) proteolytic cleavage of the propeptide does occur in the Golgi apparatus, and (2) the proteinase involved does not discriminate between glycosylated and deglycosylated precursors.

The effect of different proteinase inhibitors on propeptide release in the Golgi apparatus was also investigated. To assess the effect of the inhibitors, the presence of propeptide-containing precursors in the Golgi apparatus membrane fragment after incubation with the inhibitor was determined by immunoblotting as shown in Fig. 8. All lanes in Fig. 8(*b*) show immunoblots with

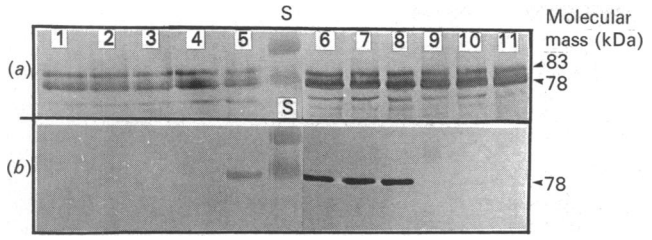

Fig. 8. Effect of inhibitors on prothrombin propeptide release

Golgi apparatus membranes were incubated in a Ca^{2+} (5 mM)-containing buffer, pH 6.0, in the presence of various inhibitors of cysteine and serine proteinases and the Ca^{2+} chelator EGTA. After incubation, the membrane fragments were subjected to SDS/PAGE in a 10% gel before immunoblotting. Immunoreactive bands shown in (*a*) and (*b*) were developed with prothrombin and anti-(prothrombin propeptide) antisera respectively. Inhibitors used: lane 1, cystatin (30 μg/ml); lane 2, E-64 (30 μg/ml); lane 3, leupeptin (30 μg/ml); lanes 4 and 5, controls; lane 6, EGTA (25 mM); lane 7, DFP (10 mM); lane 8, *p*-APMSF (1 mM); lane 9, benzamidine (1 mM); lane 10, aprotinin (30 μg/ml); lane 11, NEM (1 mM). Lane S contains prestained standard proteins. The two control lanes have membrane fragments from incubations stopped at zero time (lane 5) and after 2 h (lane 4).

the anti-propeptide antiserum. The corresponding lanes in Fig. 8(*a*) contain identical samples, but all immunoblots in this panel were developed with antiserum to prothrombin. Lanes 1, 2 and 3 are immunoblots of incubations with cystatin, E-64 and leupeptin respectively. None of these cysteine proteinase inhibitors prevented release of the propeptide. Lanes 4 and 5 are immunoblots of controls incubated for 2 h and zero time respectively. The Ca^{2+} chelator EGTA (lane 6) and the serine proteinase inhibitors DPF (lane 7) and *p*-APMSF (lane 8) strongly inhibited propeptide cleavage. However, no detectable inhibition was seen with benzamidine (lane 9), aprotinin (lane 10) or NEM (lane 11). These data demonstrate that a Ca^{2+}-dependent serine proteinase releases the propeptide from the prothrombin precursors in the Golgi apparatus.

DISCUSSION

This paper presents evidence that the propeptide carboxylase recognition site is present in late processing forms of the coagulation factor prothrombin. Data are also presented which show that vitamin K-dependent carboxylase activity is present in our preparation of the Golgi apparatus. An important question is whether this activity is a true Golgi-apparatus-located activity or results from contamination with particles from the endoplasmic reticulum in our Golgi apparatus preparation. Indeed, our Golgi apparatus preparation had 2–3% of the total glucose-6-phosphatase activity recovered from the sucrose density gradient. It has been suggested that endoplasmic reticulum marker enzyme activities also are endogenous activities of the Golgi apparatus [10,29]. Thus, based on marker enzyme activities, it is difficult to draw any conclusion about whether or not the Golgi apparatus preparation was free of contaminating microsomes. More convincing data supporting the idea of a carboxylase enzyme(s) in the Golgi apparatus came, however, from the experiment with peptides I and II from the *N*-terminal and the *C*-terminal part respectively of the Gla region of prothrombin when these peptides were used as substrates for the carboxylase. Indeed, the finding of an apparent difference in the preference for one over the other of these peptides as substrate for the carboxylase in the endoplasmic reticulum and the Golgi apparatus raises questions about the existence of different γ-carboxylation sites in the secretory pathway with different specificities for various parts of the Gla region.

As also has been shown by others [27], sucrose-density-gradient centrifugation partially separated the Golgi stack into cis- and trans-Golgi-enriched fractions. The carboxylase activity associated with our Golgi apparatus preparation was clearly enriched in fractions also enriched in cis-Golgi cisternae, suggesting that the γ-carboxylation site in late processing is present in the cis-Golgi part of the secretory pathway. However, the final confirmation of this putative cis-Golgi γ-carboxylation site must wait until the carboxylase can be localized immunohistochemically in the secretory pathway.

Our preparation of the Golgi apparatus was a combination of two published procedures, each claiming to yield a highly purified Golgi preparation. The preparation does contain endo H-sensitive prothrombin precursors, which raises question about whether pre-Golgi particles were present in our preparation. It is more likely, however, that the majority of the endo H-sensitive proteins were derived from cis-Golgi particles in our preparation. As shown by Lodish *et al.* [30], asparagine-linked glycoproteins become endo H-insensitive first after modification by mannosidase II in the medial-Golgi compartment. Electron microscopy examination of the isolated Golgi apparatus did not reveal any observable contamination with microsomal vesicles, which indeed suggests that the bulk of prothrombin precursors were derived from the Golgi apparatus. Our studies on these precursors provided additional support for the idea that γ-carboxylation is also a late processing event, as (1) deglycosylation of the Golgi apparatus precursors with recombinant *N*-Glycanase was shown to result in prothrombin precursors that were more negatively charged than corresponding precursors found in microsomes, and (2) thermal decarboxylation suggested that Gla residues may have contributed to the extra negative charges.

The data on endoproteolytic cleavage of the prothrombin precursor in the Golgi apparatus for release of the propeptide are consistent with the subcellular location of endoproteinases involved in similar processing of a variety of secretory proteins [31]. This family of endoproteinases, which cleave specifically after paired basic amino acid residues, has also been shown to be involved in the conversion of single-chain Factor X and proteins S to their two-chain forms [32]. Thus, in liver, this proteinase(s) has an important function in maintaining a normal haemostatic mechanism. Indeed, a haemophilia B type bleeding disorder has been described [33] in which the propeptide has not been cleaved from the Factor IX precursor due to an Arg-1 to Ser-1 mutation at the cleavage site. We show in this paper that the endoproteinase responsible for prothrombin propeptide release is a Ca^{2+}-dependent serine proteinase(s). The data on the characteristics of the endoproteinase(s) are identical to those reported by Oda & Ikehara [28], which describe the endoproteinase involved in conversion of proalbumin to albumin. The enzyme has characteristics that are similar to those of the mammalian KEX2-like gene product furin [34]. This Ca^{2+}-dependent subtilisin-like serine proteinase is present in $HepG_2$ cells and has been shown in transfected cells to process prohormones at a paired basic amino acid sequence [34].

This work was supported by NIH grant HL-32070. We thank Angela Higgins for typing this manuscript.

REFERENCES

1. Suttie, J. W. & Jackson, C. M. (1977) Physiol. Rev. **57**, 1–70
2. Furie, B. & Furie, B. (1990) Blood **75**, 1753–1762
3. Walz, D. A., Hewett-Emmett, D. & Guillin, M.-C. (1986) in Prothrombin and other Vitamin K Proteins (Seegers, W. A. & Walz, D. A., eds.), vol. 1, pp. 125–160, CRC Press, Boca Raton, Florida

4. Suttie, J. W. (1985) Annu. Rev. Biochem. **54**, 459–477
5. Ulrich, M. M. W., Furie, B., Jacobs, M. R., Vermeer, C. & Furie, B. C. (1989) J. Biol. Chem. **263**, 9697–9702
6. Hubbard, B. R., Ulrich, M. M. W., Jacobs, M., Vermeer, C., Walsh, C., Furie, B. & Furie, B. C. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6893–6897
7. Knobloch, J. E. & Suttie, J. W. (1987) J. Biol. Chem. **262**, 15334–15337
8. Wallin, R. & Martin, L. F. (1987) Biochem. J. **241**, 389–396
9. Dallner, G. (1974) Methods Enzymol. **31**, 191–201
10. Croze, E. M. & Morré, J. (1984) J. Cell. Physiol. **119**, 46–57
11. Fleischer, B. (1974) Methods Enzymol. **31**, 180–191
12. Berger, E. G. & Hesford, F. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4736–4739
13. Reitman, M. L. & Kornfeld, S. (1981) J. Biol. Chem. **256**, 11977–11980
14. Deutscher, S. L., Creek, K. E., Merion, M. & Hirschberg, C. B. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3938–3942
15. Noordlie, R. C. & Arion, W. J. (1966) Methods Enzymol. **9**, 619–625
16. Wallin, R. & Martin, L. F. (1988) J. Biol. Chem. **263**, 9994–10001
17. Degen, S. J. F. & Davie, E. W. (1987) Biochemistry **26**, 6165–6177
18. Atassi, M. Z. (1986) Methods Enzymol. **121**, 69–95
19. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20. O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021
21. Wallin, R., Culp, E., Coleman, D. B. & Goodman, S. R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4095–4099
22. Brennan, S. O. & Peach, R. J. (1988) FEBS Lett. **229**, 167–170
23. Poster, J. W. & Price, P. A. (1979) J. Biol. Chem. **254**, 431–436
24. Sadowski, J. A., Esmon, C. T. & Suttie, J. W. (1970) J. Biol. Chem. **251**, 2770–2776
25. Novoa, E., Seegers, W. H. & Hassovna, H. I. (1976) Prep. Biochem. **6**, 307–338
26. Paulson, J. C. & Colley, K. J. (1989) J. Biol. Chem. **264**, 17615–17618
27. Trinchera, M. & Ghidoni, R. (1989) J. Biol. Chem. **264**, 15766–15769
28. Oda, K. & Ikehara, Y. (1988) J. Biochem. (Tokyo) **104**, 159–161
29. Howell, K., Ito, A. & Palade, G. E. (1978) J. Cell Biol. **79**, 581–589
30. Lodish, H. L., Kong, N., Hirani, S. & Rasmussen, J. (1987) J. Cell Biol. **104**, 221–230
31. Loh, Y. P. (1987) Ann. N.Y. Acad. Sci. **493**, 292–307
32. Foster, D., Sprecher, C. A., Holly, R. D., Gambee, J. E., Walker, K. M. & Kumar, A. A. (1990) Biochemistry **29**, 347–354
33. Diuguid, D. L., Rabiet, M.-J., Furie, B. C., Liebman, H. A. & Furie, B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5803–5807
34. Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. & Thomas, G. (1990) J. Cell Biol. **111**, 2851–2859

Received 20 November 1990/7 February 1991; accepted 19 February 1991