Propeptide recognition by the vitamin K-dependent carboxylase in early processing of prothrombin and factor X

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Precursors of vitamin K-dependent proteins are synthesized with a propeptide that is believed to target these proteins for γ-carboxylation by the vitamin K-dependent carboxylase. In this study synthetic propeptides were used to investigate γ-carboxylation of the prothrombin and factor X precursors in rat liver microsomes. The extent of prothrombin processing by the carboxylase was also investigated. Antisera raised against the human prothrombin and factor X propeptides only recognized precursors with the respective propeptide regions. The data demonstrate structural differences in the propeptide region of the prothrombin and the factor X carboxylase substrates which raises questions about the hypothesis of a common propeptide binding site on the carboxylase for all precursors of vitamin K-dependent proteins. The hypothesis of separate binding sites is supported by data which demonstrate differences in binding of the prothrombin and factor X precursors to membrane fragments from rough and smooth microsomes. γ-Carboxylation of the prothrombin precursors in vitro was investigated with conformational specific antibodies raised against a portion of the Gla (γ-carboxyglutamic acid) region extending from residue 15 to 24. The synthetic peptide used as antigen contains three of the ten potential Gla sites in prothrombin. It is shown that these antibodies do not recognize mature prothrombin but recognize the decarboxylated protein. It is also demonstrated that the epitope is Ca^{2+}-dependent. The antibodies were used to assess γ-carboxylation of the prothrombin precursor in membrane fragments from microsomal membranes. The results suggest that microsomal γ-carboxylation does not involve Glu residues 16, 19 and 20 of the Gla region.

INTRODUCTION

Vitamin K-dependent clotting factors are secretory glycoproteins that are modified post-ribosomally by the vitamin K-dependent carboxylase [1,2]. These clotting factors are synthesized with a signal peptide for translocation [3] but have, in addition, a propeptide that extends from the N-terminus of the mature proteins [3–5]. Currently it is believed that the propeptide is the recognition site which targets these secretory glycoproteins for γ-carboxylation by the vitamin K-dependent carboxylase [6]. The carboxylase converts newly synthesized precursors of the clotting factors into γ-carboxylated proteins in a reaction which requires reduced vitamin K_1 (vitamin K_1H_2), CO_2 and O_2 [7]. The finished proteins have 10–12 γ-carboxyglutamic acid (Gla) residues which reside in the N-terminal part of the proteins [8].

Although much has been learned about the γ-carboxylation reaction, the mechanism for protein processing by the vitamin K-dependent carboxylase remains an unsolved and challenging problem. Newly synthesized precursors of these proteins are sorted by the carboxylase to fulfil γ-carboxylation of a predetermined number of glutamic acid residues that, in the prothrombin molecule for instance, are distributed over a protein sequence of 32 amino acids [4]. Failure to complete the γ-carboxylation events results in the secretion into blood of undercarboxylated forms of the clotting factors, and these factors no longer function optimally in the coagulation system because of impaired Ca^{2+} binding [1]. It has been shown that in vitamin K deficiency and after coumarin drug administration, undercarboxylated precursors of these proteins accumulate in the endoplasmic reticulum (ER) of the liver cell [9]. Several precursors of prothrombin with isoelectric points in the pI range 7.2–5.5 have been described in microsomes from warfarin-treated

rats, which suggests that processing of carboxylase substrates in various stages of γ-carboxylation is being halted by the drug [10]. Experiments in vivo have shown that this pool of unfinished precursors will serve as a substrate pool for the carboxylase when vitamin K is again available as a cofactor for the reaction. On the other hand, only a small fraction of the available precursor pool is γ-carboxylated in vitro when excess cofactor is added to the test system [2,11]. These observations suggest that additional processing events, that so far have not been reconstituted in vitro, do occur in vivo.

The long-term goal of this study is to understand protein processing by the vitamin K-dependent carboxylase. In this paper we present data on early processing of the prothrombin and factor X precursors by the carboxylase in an in vitro system prepared from rat liver.

MATERIALS AND METHODS

Preparation of rat liver microsomes

Male Sprague–Dawley rats (250–300 g; Zivic Miller Laboratories, Zelienople, PA, U.S.A.) were used for the experiments. Rats treated with warfarin were given a single intraperitoneal injection of the drug (30 mg/kg) 24 h before they were killed. Rats were anaesthetized with pentobarbital and killed by decapitation, and livers were removed for preparation of microsomes as described previously [2]. Microsomes were stored in liquid N_2.

Preparation of rough and smooth microsomes

Rough and smooth microsomes were prepared by using a sucrose/CsCl gradient as described by Dallner [12]. Microsomes

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were suspended in 250 mM-sucrose/5 mM-CsCl, and 7.0 ml of the suspension was layered above 5 ml of 1.3 M-sucrose/5 mM-benzamidine/15 mM-CsCl. Centrifugation was carried out at 4 °C in a SW 41 rotor at 39000 rev./min ($r_{av.} = 11.27$ cm) for 3 h. The pellet (rough microsomes) and the particles sedimenting in the interface (smooth microsomes) [12] were collected in different tubes, resuspended in 250 mM-sucrose/25 mM-imidazole/5 mM-benzamidine, pH 7.2, and pelleted again by centrifugation at 10000 g for 60 min.

Extraction of microsomes with detergent

Microsomes were suspended in 0.5% CHAPS/1 mM-dithiothreitol (DTT)/5 mM-benzamidine/2.5 mM-imidazole, pH 7.2 (imidazole/CHAPS buffer), to a protein concentration of 35 mg/ml. Proteins that were soluble in this buffer and the unsolubilized membrane particles in the suspension were separated by centrifugation at 100000 g for 60 min. The pellet consisting of the unsolubilized membrane particles was washed once in the imidazole/CHAPS buffer by resuspending the pellet in the buffer (8 mg of protein/ml) and subjecting it to a second centrifugation at 100000 g for 60 min. The first supernatant and the final pellet were used in the experiments and are referred to as microsomal luminal content and membrane fragments respectively.

Carboxylase activity measurements

The microsomal membrane fragments resulting from extraction of microsomes with detergent were resuspended in the imidazole/CHAPS buffer (16 mg of protein/ml) and used for carboxylase assays. Incubations contained 1 mM-$MnCl_2$, 5 mM-DTT, 2 mM of the pentapeptide carboxylase substrate Phe-Leu-Glu-Glu-Leu, 20 μCi of $NaH^{14}CO_3$/ml and 100 μg of chemically reduced vitamin K_1H_2/ml. Activity was determined as CO_2 incorporation into the synthetic peptide and the assay was carried out as described previously [13]. Incorporation of $^{14}CO_2$ into endogenous microsomal protein was measured in incubations identical with those used for peptide carboxylation, except that the pentapeptide was omitted. The ^{14}C-labelled microsomal proteins were prepared for scintillation counting as described [14].

Peptide synthesis

The peptides RECVEETCSY (peptide I), HVFLAPQARSLL-QRVRRA (peptide II), and ESLFIRREQANNILARVTRA (peptide III) were synthesized by Bachem Fine Chemicals, Torrence, CA, U.S.A. Peptide purity was guaranteed by the supplier to be > 99% pure, based on their h.p.l.c. analyses in three different chromatographic systems.

Antisera

Antisera against the synthetic peptides II and III were raised in rabbits (females, New Zealand White, 10 weeks old) as described by Atassi [15]. Peptide II and peptide III (500 μg) were 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 peptide in Freund's incomplete adjuvant.

Peptide I was coupled to keyhole-limpet haemocyanin as described by Chattopadhyay & Fair [16]. A 300 μg portion of the complex was given intradermally to a rabbit as described for peptides II and III. A booster injection of 300 μg of the complex in Freund's incomplete adjuvant was given after 4 weeks.

Blood was drawn 1 week after the last injection. Serum was stored at −20 °C. Pre-immune serum was obtained by drawing 10 ml of blood from the rabbit before immunization. Rabbit

antisera against rat plasma prothrombin and factor X were obtained as described [2].

Electrophoresis and fluorography

One-dimensional SDS/PAGE. SDS/PAGE was carried out according to Laemmli [17] in 10% gels. Before electrophoresis, all samples were boiled for 2 min in the presence of 5% (v/v) mercaptoethanol and 2% (v/v) SDS. Standard proteins used for molecular mass determinations were either low-molecular-mass ^{14}C-methylated standard proteins purchased from Amersham Corp., Arlington Heights, IL, U.S.A., or standard proteins purchased from BioRad, Richmond, CA, U.S.A. Gels selected for fluorography were treated as described in [2]. Lanes with radioactively labelled proteins appearing on the photographic film were scanned with a Hoffer Scientific Instruments scanning densitometer. Specific labelling of the prothrombin carboxylase substrate was calculated as units of the scanned integrated area/μg of prothrombin precursor applied to the gel. Gels were stained and destained as described by Fairbanks et al. [18].

Two-dimensional PAGE. Two-dimensional PAGE of immunocomplexes was carried out according to a modification of the O'Farrell method [19] as described in [20]. Immunocomplexes isolated with *Staphyloccus aureus* Protein A particles were released from the *Staphylococcus* particles with the urea/Triton X-100 sample buffer used for this system [20].

Immunochemical methods

Western blotting. Proteins separated in SDS/PAGE gels were transferred to Immobilon-P membranes (Millipore Corp.) in a BioRad Trans-Blot cell apparatus as described [2]. Before incubation with antiserum, the Immobilon membrane was treated with 5% milk fat (Blotto) as described in [2]. Antisera against the synthetic peptides, rat plasma prothrombin and factor X were added to Blotto in the proportions described in the Figure legends. Immunoreactive proteins attached to the Immobilon membrane were visualized after horseradish peroxidase reduction of 4-chloro-1-naphthol [21]. Immunoblotting in the presence of 20 mM-EDTA was carried out in buffer containing 5% BSA instead of milk fat.

Immunodot blotting. For quantitative estimation of antigens, portions of 1–2 μl of each sample were spotted on nitrocellulose membranes and treated with antiserum as described in [2]. The nitrocellulose membrane was then reacted with 0.1 μCi of ^{125}I-Protein A/ml and prepared for scintillation counting as described [21]. Purified rat plasma prothrombin and factor X were used as standard proteins for estimation of antigens in microsomal samples. When propeptide antisera were used, the microsomal prothrombin and factor X precursors were used as standard proteins.

Decarboxylation of γ-carboxyglutamic acid. Decarboxylation of Gla in proteins was carried out as described by Poster & Price [22]. Prothrombin (250 mg; dried) was decarboxylated by heating *in vacuo* at 110 °C for 6 h.

Materials

Vitamin K_1, CHAPS, di-isopropyl fluorophosphate (DFP) and Freund's adjuvants were from Sigma, St. Louis, MO, U.S.A. The vitamin was reduced to vitamin K_1H_2 as described by Sadowski et al. [23]. The pentapeptide substrate Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals, Tucson, AZ, U.S.A. $NaH^{14}CO_3$ (60 mCi/mmol) was from ICN Biochemicals. *Staphylococcus aureus* Protein A (Pansorbin) was from

Calbiochem, San Diego, CA, U.S.A. ^{125}I-Protein A (88 μCi/μg) was from NEN Research Products, Boston, MA, U.S.A. Prothrombin and factor X were purified from rat plasma as described by Novoa *et al.* [24]. Prothrombin purified from human plasma was purchased from Sigma. Antiserum against human prothrombin was purchased from Dako Corporation, Santa Barbara, CA, U.S.A. Protein was measured with the BioRad protein assay.

RESULTS

Three custom-made peptides with sequences covering the propeptide regions of human prothrombin and factor X and the first disulphide loop in prothrombin [8] were used in these studies, and their primary structures are given in Fig. 1. When present at 10–20 μM in the carboxylase test system the synthetic factor X and prothrombin propeptides were both found to stimulate carboxylase about 2-fold. The experiment showed that the synthetic propeptides both established conformations which were recognized by the rat vitamin K-dependent carboxylase. Thus the peptides could be used as analytical tools to investigate processing of the prothrombin and the factor X precursors in our rat vitamin K-dependent carboxylation system.

As shown in Fig. 2, antibodies raised against the two synthetic propeptides recognized these peptide structures in the prothrombin (Fig. 2a, lane d) and the factor X precursors (Fig. 2b, lane d) that are present in rat liver microsomes. The prothrombin and
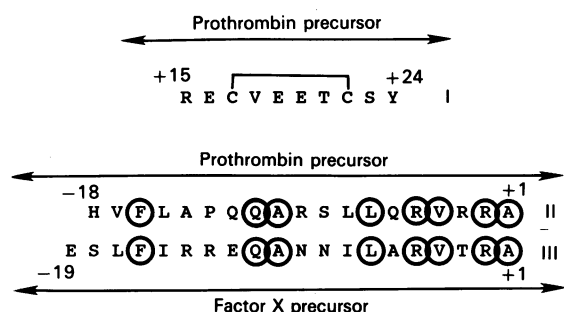

Prothrombin precursor

+15 ⎡   ⎤ +24
R E C V E E T C S Y   I

Prothrombin precursor

−18   +1
H V(F)L A P Q(Q)(A)R S L(L)Q(R)(V)R(R)(A) II

E S L(F)I R R E(Q)(A)N N I(L)A(R)(V)T(R)(A) III
−19   +1

Factor X precursor

Fig. 1. Primary structures of synthetic peptides I–III

The sequences of peptide I and II extend from residues +15 to +24 and from −18 to +1 respectively of the human prothrombin precursor sequence [4]. Peptide III extends from residues −19 to +1 of the human factor X precursor sequence [8]. The two cysteine residues (C) in peptide I are connected by a disulphide bridge.
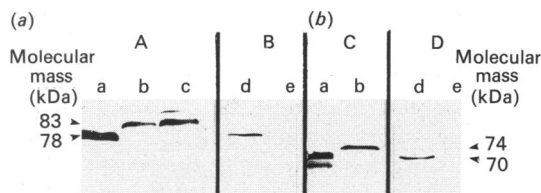
the factor X precursors were also identified with antibodies raised against the purified plasma factors and the precursors appeared on the Western blots with molecular masses of 78 (Fig. 2a, lane a) and 70 kDa (Fig. 2b, lane a) respectively. As seen in Fig. 2(b) (lane a), the factor X antibodies also recognized a protein with lower molecular mass than the 70 kDa factor X precursor, but this protein was not recognized by the factor X propeptide antiserum (Fig. 2b, lane d) and thus may represent a degradation product of the factor X precursor. Plasma prothrombin (Fig. 2a, lanes b and c) and plasma factor X (Fig. 2b, lane b) appeared on the blots with higher molecular masses than their respective microsomal precursors. The differences in apparent molecular mass between the plasma factor and microsomal precursor were 5 kDa for prothrombin and 4 kDa for factor X (see Fig. 2). These increased molecular masses of the plasma factors have been shown to result from glycosylation of the proteins [10]. As expected, the prothrombin and factor X propeptide antisera did not recognize the plasma factors (see Figs. 2a and 2b, lanes e).

As shown in Fig. 2(a) (lane d), no immunoreaction was seen between the 70 kDa factor X precursor and the prothrombin propeptide antiserum. Likewise, no immunoreaction was seen between the 78 kDa prothrombin precursor and the factor X propeptide antiserum (Fig. 2b, lane d). Thus, although significant sequence similarity exists between the prothrombin and factor X propeptides (see Fig. 1), the prothrombin propeptide antiserum was unable to recognize the propeptide region of the factor X precursor, and similarly the factor X propeptide antiserum did not recognize the propeptide region of the prothrombin precursor. These data show that the polypeptide structures in the propeptide region of the factor X and prothrombin precursors are different, suggesting that there could be differences in the interactions of these substrates with the carboxylase enzyme.

The prothrombin and factor X carboxylase substrates have been identified previously [2] as the ^{14}C-labelled 78 and 70 kDa bands respectively shown on the fluorogram in Fig. 3. Using fluorography to identify and quantify labelling of the prothrombin and factor X carboxylase substrates, we investigated whether or not the synthetic propeptides interfered with binding of these substrates to the carboxylase. In one set of experiments, portions of membrane fragments were used for vitamin K-dependent ^{14}C-labelling in the presence of various concentrations of the prothrombin propeptide (Fig. 3, lanes a–d, f). Lane e has protein from a portion that was incubated in the absence of vitamin K_1H_2. Densitometric scanning of the fluorogram showed

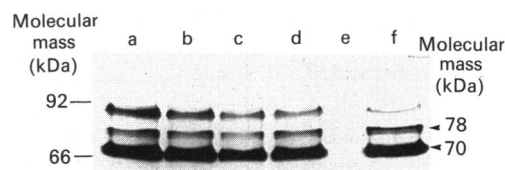**Fig. 2. Western blotting of prothrombin and factor X antigens in rat liver microsomes and rat plasma**

Lanes a and d in (a) and (b) contain 100 μg of protein from warfarin microsomes. Lane b contains 2.5 μl of rat plasma and lanes c and e contain 5 μl of rat plasma. Sections A, B, C and D were treated with rat prothrombin antiserum (dilution in Blotto 1:750), prothrombin propeptide antiserum (dilution 1:500), rat factor X antiserum (dilution 1:750) and factor X propeptide antiserum (dilution 1:500) respectively. Apparent molecular masses of immunoreactive bands are indicated.

Fig. 3. [14C]Carboxylation of the prothrombin carboxylase substrate in the presence of excess propeptide

Vitamin K-dependent ^{14}C-labelling of proteins in membrane fragments from warfarin-microsomes was carried out in the absence (lane a) and the presence of 2.5 (lane b), 10 (lane c), 25 (lane d) or 200 μM of the prothrombin propeptide (peptide II). Lane e contains protein from membrane fragments that were labelled in the absence of vitamin K_1H_2. The fluorogram shows ^{14}C-labelling of the prothrombin (78 kDa) and the factor X (70 kDa) carboxylase substrates. The amount of prothrombin antigen present in the various lanes was 30 ng for lanes a, b, e and f, and 22 ng for lanes c and d.

no change in specific ^{14}C-labelling (see the Materials and methods section) of the prothrombin precursor whether labelling had been carried out in the absence (lane a) or in the presence (lanes b–d and f) of the propeptide. Similar results were obtained with the factor X propeptide (results not shown). The experiment demonstrated that the propeptides were unable to compete with the prothrombin and factor X substrates for binding to the carboxylase, thus making less substrate available for γ-carboxylation. This was confirmed by measuring the effect of the prothrombin and factor X propeptides on γ-carboxylation of proteins in the membrane fragments. There was no difference in total protein γ-carboxylation between samples containing the prothrombin (1690 c.p.m./mg) or the factor X (1710 c.p.m./mg) propeptides and the control (1720 c.p.m./mg). We conclude from these experiments that neither propeptide interfered with γ-carboxylation of the prothrombin and factor X precursors.

We have presented data which suggest that the prothrombin and factor X carboxylase substrates are bound to different proteins in the ER [2]. We found that warfarin treatment of rats resulted in increased membrane binding of the factor X carboxylase substrate, whereas the membrane concentration of the prothrombin carboxylase substrate was unaffected by the drug [2]. In this study we measured the effect of the drug on binding of the prothrombin and factor X precursors to membrane fragments from rough and smooth microsomes. The concentration of antigens in membrane fragments and in detergent extracts of rough and smooth microsomes were quantified by dot-blotting using antisera against the propeptides for recognition of the antigens. As shown in Fig. 4(b), the prothrombin precursor concentration in the rough membrane was unaffected by administration of warfarin to the rats. However, in the detergent extract of these particles, which included the luminal content of rough ER, a 2.5-fold increase in the prothrombin precursor concentration was measured (Fig. 4b). On the other hand, as shown in Fig. 4(a), warfarin treatment of the rats had no effect on the prothrombin precursor concentration in smooth microsomes. The factor X precursor concentration increased in both the rough (Fig. 4d) and the smooth (Fig. 4c) membranes, and also in the detergent extracts of these microsomes, as a result of warfarin administration to the rats. Thus localization differences in the ER membrane with regard to binding of the prothrombin and factor X carboxylase substrates were apparent from these experiments.

We also investigated the extent to which the vitamin K-dependent carboxylase was able to γ-carboxylate the various forms of the endogenous prothrombin precursors that were present in our 'in vitro' system. Antibodies raised against peptide I (see Fig. 1) were used for these studies. As shown in Fig. 5, we could demonstrate that these antibodies did not recognize mature prothrombin (lane e), but recognized this protein after its Gla residues had been converted into Glu residues by decarboxylation (Fig. 5, lane d). A similar conformational change was seen when Ca^{2+} was removed from prothrombin with EDTA (Fig. 5c). The peptide I antibodies also recognized prothrombin precursor(s) in rat liver microsomes (Fig. 5, lane c), demonstrating the presence of precursors with incomplete γ-carboxylation of the first disulphide loop in prothrombin. Also shown in Fig. 5 (lanes a and b) are the immunoreactions of human prothrombin and decarboxylated human prothrombin with antibodies against human prothrombin. In addition to human prothrombin (72 kDa) [8], the antibodies also recognized proteins with lower molecular mass (Fig. 5, lanes a and b). These bands probably represent activation fragments of prothrombin [8] which are present in the commercial prothrombin preparation that we used for this experiment. The experiments shown in Fig. 5 demonstrate that we could use the peptide I antibodies to assess the ability of
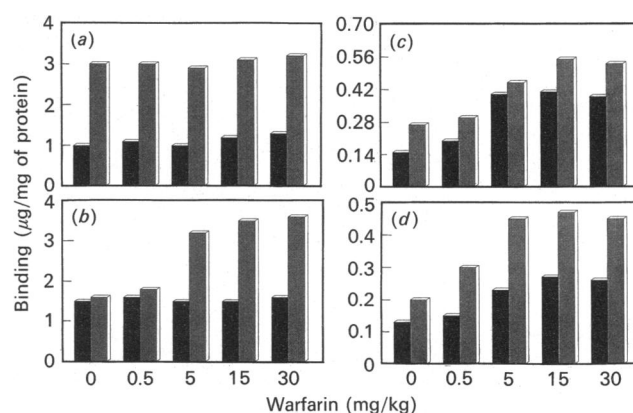

Fig. 4. Effect of warfarin administration to rats on binding of the prothrombin and factor X precursors to rough and smooth ER fragments

Membrane fragments and the luminal content from rough (b and d) and smooth (a and c) liver microsomes were prepared from rats treated with the various concentrations of warfarin indicated. The precursor concentration in membrane fragments (■) and the luminal content (▨) were determined with the prothrombin (a and b) and factor X (c and d) propeptide antisera in a quantitative immunodot-blot assay. Each data point is the average of four parallel determinations differing by less than 10 %.
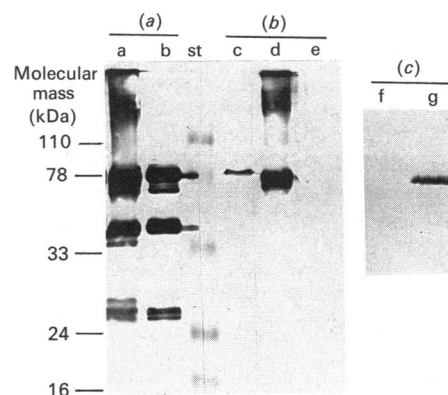

Fig. 5. Western blotting with peptide I antiserum

Lanes a and d contain 1 μg of decarboxylated human prothrombin; lanes b and e contain 1 μg of commercially available purified human prothrombin (see the Materials and methods section); lane c contains 25 μg of protein from warfarin microsomes; lanes f and g contain 5 μl of human plasma; lane st contains prestained standard proteins. (a) Proteins that reacted with human prothrombin antiserum; (b) and (c) show proteins that reacted with the peptide I antiserum. The Western blot shown in (c) was developed in the absence (lane f) and presence (lane g) of 20 mM-EDTA.

our 'in vitro' system to convert the Glu residues in the first disulphide loop of prothrombin into Gla residues. The experiment was carried out by comparing peptide I antibody binding to membrane fragments from rough and smooth microsomes before and after γ-carboxylation of proteins in vitro that were present in these fragments. As shown in Fig. 6(a), we measured more antibody binding per mg of protein to the prothrombin precursor in membranes from smooth (S) than from rough (R) microsomes. Antibody binding was not affected, however, by γ-carboxylation in vitro (see Fig. 6b). These data suggested that the carboxylase in our 'in vitro' system was unable to complete γ-carboxylation of the disulphide loop.

We also investigated by two-dimensional SDS/PAGE the prothrombin precursors that were ^{14}C-labelled in our 'in vitro' γ-
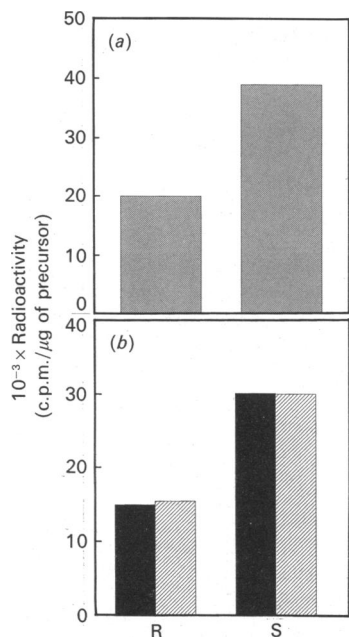
Fig. 6. Peptide I antibody binding to prothrombin precursors in membrane fragments from rough and smooth ER

Binding of peptide I antibodies to membrane fragments from rough (R) and smooth (S) warfarin microsomes was estimated by immunodot-blotting. (a) Binding of ^{125}I-Protein A to proteins in soluble rough and smooth ER fragments when immobilized on nitrocellulose. (b) Binding of ^{125}I-Protein A before (▨) and after (■) γ-carboxylation of protein present in the fragments. The data are the means of four parallel determinations differing by less than 10%.
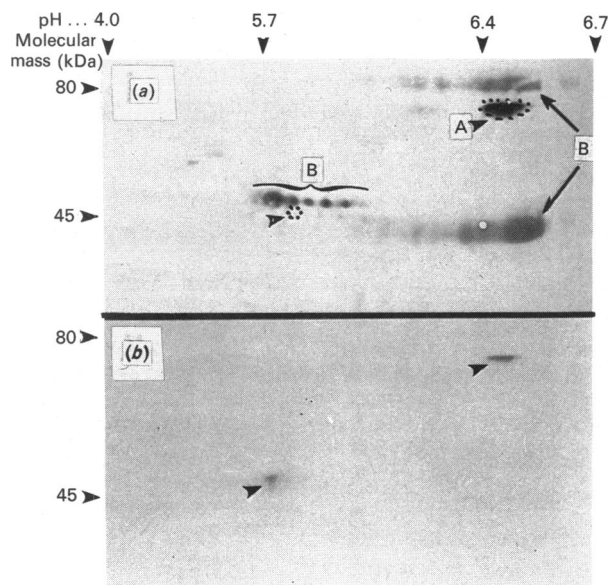
Fig. 7. Two-dimensional SDS/PAGE of prothrombin carboxylase substrates

Membrane fragments from warfarin microsomes were ^{14}C-γ-carboxylated and reacted with rat prothrombin antiserum for isolation of the prothrombin precursors as described in the Materials and methods section. Immunocomplexes bound to the Pansorbin particles were released with the sample buffer used for isoelectric focusing and subjected to two-dimensional SDS/PAGE. Proteins were transferred to an Immobilon P membrane for immunoblotting with rat prothrombin antiserum (a); (b) shows an autoradiogram of the immunoblot in (a). The positions of the radioactive spots are indicated by the broken lines in (a). The arrows marked 'B' point to background staining caused by contaminating material in the sample.

carboxylation system. Fig. 7(a) shows proteins from the two-dimensional SDS/PAGE gel after transfer to an Immobilon P membrane for immunoblotting with prothrombin antiserum. Unspecific staining of the blot resulting from IgG and contaminating protein released from the Staphylococcus aureus particles is identified by the letter B in Fig. 7(a). The heaviest peroxidase staining was associated with a protein of apparent isoelectric point (pI) of 6.45–6.50 ('A' in Fig. 7a). An autoradiogram of the Western blot in Fig. 7(a) is shown in Fig. 7(b). Two radioactive spots were found and their location on the blot is indicated by the broken lines in Fig. 7(a). As can be seen, only the pI 6.45–6.50 peroxidase-stained spot was found to be radioactive. The other ^{14}C label was clearly separated from the peroxidase-stained spots, but its protein association has not yet been identified. This experiment identified the prothrombin carboxylase substrates as protein(s) with pI 6.45–6.50, which is significantly different from that of mature rat prothrombin (pI 5.3–5.7) [11].

A quantitative estimate of the extent of γ-carboxylation in vitro of the prothrombin precursor pool in membranes from rough and smooth microsomes was also made. For this, rough and smooth membranes were ^{14}C-γ-carboxylated, absorbed with excess prothrombin antiserum and immunocomplexes were analysed by fluorography as previously described [2]. It was found (results not shown) that specific ^{14}C labelling of the prothrombin precursor pool in smooth membranes was only 20% of labelling of this pool in the rough membranes. These data demonstrate that γ-carboxylation of the prothrombin precursor in vitro is mainly a rough-ER event.

DISCUSSION

Synthetic propeptides have played a major role in our current understanding of γ-carboxylation of vitamin K-dependent proteins [5]. The prothrombin and factor X propeptides have been shown not only to have binding affinity for the carboxylase [5] but also to modulate enzyme activity, suggesting an additional regulatory role for the propeptides in processing of its substrates [25]. Recently the carboxylase has been purified successfully from bovine liver microsomes by biospecific affinity chromatography on a propeptide-containing matrix [26].

Most research on the γ-carboxylation reaction has been conducted with synthetic peptides that are accepted as substrates by the carboxylase. However, very little is known about γ-carboxylation and processing of its natural protein substrates. In this work the natural protein substrates were the focus for investigation. The analytical tools used in the work were synthetic peptides made against sequences taken from human prothrombin. This was necessary, since the primary structures of the rat prothrombin and factor X precursors are unknown. Antibodies raised against the synthetic peptides recognized the human as well as the rat proteins, which demonstrates that these peptide structures are highly conserved.

If the carboxylase interacts directly with its protein substrates, the binding site on the carboxylase for the propeptide would be expected to have the capability to interact with the different propeptides present in all vitamin K-dependent proteins targeted for γ-carboxylation. Our data question this model. As shown, the propeptide regions of the prothrombin and the factor X precursors share no structural features that result in common epitopes recognized by the propeptide antibodies. One could of course argue that the antibodies were raised to sequences unique to the prothrombin and factor X propeptides, but not necessarily to a conformation or sequence that all propeptides might have in common and which could be recognized by the carboxylase.

However, our hypothesis is also supported by published data. Hubbard et al. [26] have reported that affinity purification of the carboxylase on a matrix with the prothrombin propeptide attached resulted in binding of only 40% of the carboxylase activity to the column resin. De Metz et al. [27] have shown that separate pools of the carboxylase exist as complexes with its prothrombin, factor X and factor IX substrates respectively. These observations would also support the existence of different pools of carboxylase enzymes with different preferences for propeptide binding. Furthermore, our data on binding of the prothrombin and factor X precursors to membrane fragments from smooth and rough microsomes after warfarin treatment of rats also support the notion of a difference in binding of the prothrombin and the factor X precursors to the membrane-bound carboxylase.

In contrast with their stimulation of peptide carboxylation, the synthetic propeptides did not affect γ-carboxylation of the pro-thrombin and factor X carboxylase substrates. Clearly the propeptides could not compete with the protein substrate for binding to the carboxylase and did not stimulate γ-carboxylation of proteins. These data would suggest that more than one site exists for interaction between the carboxylase and its protein. Indeed, it has been suggested by Price et al. [28] that an additional consensus sequence in the N-terminal region of vitamin K-dependent proteins may also be involved in carboxylase protein substrate binding.

It appears from our data that microsomal γ-carboxylation of the prothrombin precursor is restricted to potential Gla sites other than the sites in the first disulphide loop of the prothrombin molecule. These include sites four and five of the ten potential Gla sites starting from the N-terminal part of the protein [4]. We feel comfortable with this conclusion, since γ-carboxylation of the total prothrombin pool in vitro has been shown to be 20–35% completed [11]. Thus the carboxylase in our system in vitro is not capable of carrying out all of the γ-carboxylation events which occur in vivo, where the entire precursor pool will be converted into mature prothrombin with 10 Gla residues. Clearly something is lost in vitro. What is lost appears to be the ability of the system to present, as substrates for the carboxylase, precursors of vitamin K-dependent proteins that are further along in processing. This view is also supported by data which show a significant decrease in γ-carboxylation of the prothrombin precursor pool in smooth microsomes. Previous work [29] has shown a large pool of more mature prothrombin precursors in smooth ER than in rough ER.

In conclusion, we have shown that processing of proteins by the vitamin K-dependent carboxylase is a complex event which apparently involves proteins and reactions not yet recognized as part of the vitamin K-dependent carboxylation system. Clearly the system needs to be understood in greater detail before a plausible model can be proposed for processing of vitamin K-dependent proteins.

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