The early stages in the biosynthesis of prothrombin

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Prothrombin precursors undergo several modifications before secretion from the liver. These include signal-peptide cleavage (Friezner-Degen et al., 1983), complex glycosylation (Mizuoche et al., 1979) and disulphide-bridge formation (Magnusson et al., 1975). Moreover, the function of plasma prothrombin is absolutely dependent upon the presence, at its N-terminus, of ten residues of γ-carboxyglutamic acid (Stenflo et al., 1974). This novel amino acid is generated in the endoplasmic reticulum of the liver cell by the vitamin K-dependent carboxylation of specific glutamic acid residues in prothrombin precursors (Olson & Suttie, 1977).

At present, the temporal sequence and mechanism of the processing events responsible for the modification and secretion of prothrombin are poorly understood. We have employed a combination of approaches in vitro to define the steps in prothrombin biosynthesis.

In order to locate the intracellular site of prothrombin synthesis, RNA was extracted (Palmiter, 1974) from endoplasmic reticulum-bound and cytosolic rat liver polysomes (fractionated according to Ikehara & Pitot, 1973). When these RNAs were used to direct protein synthesis in a reticulocyte lysate (Pelham & Jackson, 1976), prothrombin was made only in response to the RNA from the membrane-bound polysomes.

In a second series of experiments a prothrombin-processing system was developed in vitro. Bovine prothrombin mRNA was partially purified by hybridization (Goldberg et al., 1979) to prothrombin complementary DNA (MacGillivray et al., 1980), cloned into the single-stranded fd phage vector fd 103 (Heremann et al., 1980). When dog pancreas microsomes were added to a reticulocyte lysate actively translating this mRNA, a major proportion of the nascent prothrombin exhibited an increased molecular mass (~90 kilodaltons, compared with ~85 kilodaltons for the protein made in the absence of microsomes). Preliminary concanavalin A-binding experiments suggest that the mobility shift may be due to the co-translational addition of mannose-rich carbohydrate to the prothrombin translation product. Moreover, the pancreatic membranes protected a significant amount (~40%) of the 90 kilodalton form against proteases added after translation, but only if the membrane structure was intact during proteolysis. Thus, some of the nascent prothrombin had segregated into the cisternae of the reticulum during translation. Since only the 90-kilodalton form was protected against proteases, the processing event responsible for the molecular mass shift may be coupled to the membrane transport of prothrombin.

The mechanistic details of the vitamin K-dependent carboxylation, including the nature of the substrate for the reaction, have remained unclear since the discovery of γ-carboxyglutamic acid in 1974. Previous studies (Souté et al., 1981) have shown that descarboxyprothrombin (from anticoagulated bovine plasma) is a poor substrate when added to the microsomal carboxylation system of Suttie (Suttie et al., 1976). In an attempt to identify the natural substrate for the carboxylase, we have purified prothrombin precursor activity 100-fold from vitamin K-deficient rat liver microsomes by the use of an anti-antithrombin immunoglobulin G-Sepharose absorbent followed by fast protein liquid chromatography on a Mono Q column (Pharmacia) (M. R. Evans, M. W. P. Sung & M. P. Esnouf, unpublished work). Two closely spaced bands were observed in the prothrombin region when the purified material was subjected to sodium dodecyl sulphate gel electrophoresis. When an electrophoretic 'blot' of this gel (Burnette, 1981) was stained by the immunoperoxidase technique (Burke et al., 1982) both bands were detected, confirming their immunological identity as prothrombin. Furthermore, in agreement with the processing in vitro results, the microsomal prothrombins contained exposed mannose or glucose

Table 1. Carboxylation of purified prothrombin precursor

<table>
<thead>
<tr>
<th>Incubation</th>
<th>d.p.m. incorporated into protein</th>
<th>d.p.m. incorporated into exogenous protein</th>
<th>Specific radioactivity (d.p.m./mg of solubilized protein)</th>
<th>Specific radioactivity (d.p.m./mg of exogenous protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No exogenous protein, solubilized microsomes, 14C added at time 0min</td>
<td>37850</td>
<td>—</td>
<td>5407</td>
<td>—</td>
</tr>
<tr>
<td>B. No exogenous protein, solubilized microsomes, 14C added at time 15min</td>
<td>19000</td>
<td>—</td>
<td>2714</td>
<td>—</td>
</tr>
<tr>
<td>C. Same as B, except prothrombin precursor (120 µg) added with 14C</td>
<td>32000</td>
<td>13000</td>
<td>4494</td>
<td>108000</td>
</tr>
</tbody>
</table>

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residues as shown by concanavalin A–peroxidase staining of corresponding blots. The purified precursors were readily carboxylated by the microsomal system (Table 1), in contrast to decarboxylated plasma prothrombin. This suggests that the precursors may contain an additional signal which promotes carboxylation. To test this idea the rat prothrombin precursors were sequenced. The N-terminus was apparently blocked, which is consistent with the possible existence of a carboxylase promoter region as mature rat prothrombin has an alanine residue at the N-terminus (Houser et al., 1977). A similar result has been obtained by Grant & Suttie (1976).

In preliminary experiments, the synthesis and secretion of prothrombin have also been monitored in isolated rat hepatocytes. The addition of tetrachloropyridinol, a specific inhibitor of vitamin K-dependent carboxylation, selectively inhibited prothrombin secretion by these cells. Hence, as expected from studies in vitro (Shah et al., 1983) precursor carboxylation appears to act as a signal for prothrombin secretion in the rat. The hepatocytes approach should be of significant use for investigating the molecular basis of this phenomenon.

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Isolation of a type VI collagen precursor from bovine elastic tissues

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Microfibrillar structures have long been recognized as ubiquitous constituents of connective tissues. The microfibrillar glycoproteins associated with elastic fibres appear to have a key role in elastogenesis but similar structures associated with collagen fibres seem to provide a connecting link between the extracellular fibres, basal lamina and cells. It has been shown to be partially collagenous and a major glycoprotein (designated MFPI) associated with elastic fibres appears to have a key role in elastogenesis but similar structures associated microfibrils could be extracted from tissues by disulphide bond reducing agent was not found necessary by glycoproteins (designated MFPI and MFPII) synthesized and extracted sequentially with the following

Porcine nuchal ligament (Sear et al., 1978, 1981a,b). Glycoprotein MFPI was shown to be partially collagenous and a major glycoprotein (designated tissue MFPI) with similar properties to the biosynthetic product identified by Sear et al. (1978, 1981a) was subsequently extracted from intact bovine nuchal ligament (Kight et al., 1984). In this communication we report the extraction of glycoprotein MFPI from several elastic tissues with markedly different contents of elastin.

Abbreviations used: SDS, sodium dodecyl sulphate; PAS, periodic acid/Schiff's.

Evidence is also presented which indicates that glycoprotein MFPI is a high M, precursor form of the highly disulphide-bonded collagen previously termed 'high-molecular-weight' (HMW)-aggregate (Furuto & Miller, 1980, 1981; Laurain et al., 1980) or 'intima collagen' (Jander et al., 1983), but now defined as type VI collagen (Jander et al., 1983; Forthmayr et al., 1983).

Bovine nuchal ligament, aorta and uterus were milled in liquid N₂ and extracted sequentially with the following buffered solutions: 50mM-Tris/HCl buffer, pH 7.4, containing 50mM-NaCl; 50mM-Tris/HCl buffer, pH 7.4, containing 0.6mM-KCl; 50mM-Tris/HCl buffer, pH8.0, containing 5mM-guanidinium chloride; and 100mM-Tris/HCl buffer, pH8.3, containing 5mM-guanidinium chloride and 50mM-dithiothreitol. All the solutions contained a cocktail of proteinase inhibitors to minimize proteolysis during the extraction procedures. The extracts obtained with 5mM-guanidinium chloride (designated G extracts) and with guanidinium chloride with a reducing agent (designated GD extracts) were extensively dialysed against water and the resulting precipitates (Gp and GDp) were separated from the soluble fractions (Gs and GDs) by centrifugation. The three tissues were also extracted with buffered NaCl and KC1 solutions as above and then digested with pepsin in 0.5m-earctic acid (E:S of 1:100) at 4°C for 48h. The solubilized collagen were fractionated to yield collagen types I, III, IV, V and VI (Abelin et al., 1982; Chambers et al., 1984). In addition, the Gp fractions were treated with pepsin as above and their susceptibilities to highly purified bacterial collagenase were tested by incubation (E:S of 1:10) in 50mM-Tris/HCl buffer, pH7.4, containing 5mM-CaCl₂, 2mM-N-ethylmaleimide and 1mM-phenylmethylsulphonyl fluoride for 24h at 37°C. SDS/polyacrylamide-gel electrophoresis was carried out according to Laemmli (1970) and polypeptides were stained with Coomassie Blue, and glycoproteins with the PAS reagent (Fairbanks et al., 1971). In some experiments polypeptides of interest were

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