ABSTRACT The mechanisms by which blood levels of prothrombin (PT) are regulated in the vitamin K-sufficient state are unknown. We have studied PT synthesis by Reuber H-35 rat hepatoma cells exposed to vitamin K and [3H]leucine in serum-free cultures. Administration to the culture system of exogenous bovine PT and rat PT was characterized by increases in endogenous PT synthesis and secretion of 2- and 3-fold, respectively. This induction required endogenous proteolytic degradation of PT. Studies conducted with bovine PT fragment 1 (residues 1–156) demonstrated up to 5-fold increases in PT synthesis. This induction was dose dependent and saturable. Addition of bovine PT chymotryptic fragments to the cells indicated that the NH2-terminal peptide of prothrombin (residues 1–42) contained the requisite structural elements for the induction. Peptide-bound γ-carboxyglutamate residues were required for the observed stimulation of PT synthesis. These results suggest that PT synthesis might be regulated physiologically by the products formed during its normal turnover and consumption during blood coagulation.

Normal coagulation requires that plasma concentrations of the clotting factors be maintained within relatively narrow ranges. An understanding of the mechanisms by which their synthesis and consumption are regulated is therefore essential. Extensive structural and enzymatic studies of several clotting factors (1, 2) have provided information concerning their activation and, thus, consumption. However, only recently has the regulation of their biosynthesis been investigated (3–10). While the essential role of vitamin K in the γ-carboxylation of glutamic acid residues in clotting factors II, VII, IX, and X has been elucidated (10), the mechanism by which the biosynthesis of these and other clotting factors are regulated when sufficient vitamin K is present is poorly understood. Several investigators (3–5) have reported that a factor present in the plasma of hypoprothrombinemic animals will induce synthesis of the vitamin K-dependent coagulation factors in normal animals. Other workers have suggested that synthesis of fibrinogen (7, 8) or prothrombin (11) is regulated by polypeptides generated from these factors during normal coagulation. Kessler and coworkers (7, 8) found that, after infusions of fibrin degradation products, fibrinogen synthesis was increased. Unfortunately, a comparable study (9) has not confirmed this observation. Munns et al. (11) postulated that prothrombin (PT) synthesis by hepatoma cells was induced by proteolytic fragments of PT produced during its extracellular degradation. These preliminary observations do not allow definitive conclusions concerning the biosynthetic regulation of clotting factors.

Because of the central role of PT in coagulation, we have continued our studies on regulation of its biosynthesis (11). Prothrombin, the plasma zymogen of thrombin, is a single polypeptide chain with an apparent molecular weight of 68,000 to 74,000. The complete amino acid sequences of bovine and human PT and parts of the rat protein are known (12–14). During coagulation, thrombin is generated from PT by the action of Factor X in the presence of Factor Va, Ca2+, and phospholipids. While the NH2-terminal, γ-carboxyglutamyl-containing portion (fragment 1-2) of PT is required for optimal physiological activation (1, 2), the fate and possible further functions of this fragment are unknown. We previously observed that cultured H-35 rat hepatoma cells synthesize more PT after exposure to vitamin K for 18 hr (11), and we proposed that part of this induction might be due to the accumulation of vitamin K-dependent peptides. We report here that addition of proteolytic fragments derived from PT will induce its synthesis in cell culture by up to 5-fold. Moreover, addition of purified PT fragments to the cells proved that the NH2-terminal γ-carboxyglutamyl-containing peptide (residues 1–42) contained the structural features responsible for this induction.

MATERIALS AND METHODS

Materials. [3H]Leucine (40–60 Ci/mmol; 1 Ci = 3.7 × 1010 becquerels) was purchased from Amersham. Phylloquinone (K1), as the water-soluble preparation Aqua Mephyton, was obtained from Merck Sharp and Dohme and prepared as reported (11, 15). Insulin and benzamidine-HCl were purchased from Eli Lilly and Calbiochem, respectively.

Preparation of Peptide Reagents. Rat PT was purified as described (14). Bovine PT, fragment 1 (residues 1–156), fragment 2 (residues 157–274), and thrombin (residues 275–582) were prepared as reported (16) from a barium citrate precipitate (17) except that chromatography on heparin linked to agarose preceded gel filtration in the PT preparation. Residues 1–42 and 46–156 were prepared according to Morita and Jackson (18). After proteolysis of fragment 1 at 1–5 mg/ml with α-chymotrypsin (1/270 wt/wt, 30 min at 37°C) the peptides were separated by chromatography using QAE-Sephadex with 0.1–0.6 M NaCl in 0.02 M Tris-HCl, pH 7.5. PT fragment 1 was thermally decarboxylated according to the protocol of Poser and Price (19). The extent of decarboxylation was determined by amino acid analysis (20) and indicated quantitative conversion of γ-carboxyglutamate to glutamate without other chemical or structural changes in the peptide. Only peptides exhibiting single bands after electrophoresis in NaDodSO4/acylamide gels (21, 22) were used as inducing agents in these studies.

Cell Culture Conditions and Assays for Induction Activity. The cell line H4-11-E-C3 used in these studies was originally obtained from Van R. Potter (University of Wisconsin, Madison). The cells were propagated in monolayer culture as previously described (11, 15). Confluent cells were maintained for 18 hr with serum-free medium containing insulin at 1 μg/ml. Vitamin K was added at 0.1 μg/ml for 1.5 hr prior to initiating

Abbreviation: PT, prothrombin.
an experiment. Protocols for induction of PT synthesis by addition of PT or fragments derived from it are described in the text and figure legends. Vitamin K was always present during subsequent medium changes. At the conclusion of the induction protocols, cells were re-fed with leucine-free, polypeptide-free medium and pulsed for 1 hr with [3H]leucine. [3H]-Labeled rat PT in the culture medium was adsorbed to and eluted from Sepharose-conjugated antibodies to rat PT as described (15, 23). The quantity of secreted rat [3H]PT was determined after electrophoresis in NaDodSO4/polyacrylamide gels (22) and expressed as the percent of the total trichloroacetic acid-precipitable radioactivity present in the culture medium.

RESULTS

Induction of PT Synthesis in Cells Exposed to Exogenous PT. Endogenous rat PT synthesis was measured after exposure of H-35 cells to bovine PT or rat PT according to the procedures outlined in the legend to Fig. 1 and Materials and Methods. Gel profiles of purified, extracellular PT revealed three peaks of radioactivity (Fig. 1); the largest comigrated with mature rat PT. The two smaller peaks migrated with apparent molecular weights of 60,000 and 35,000 and contained 35-40% of the total radioactivity. We have previously demonstrated that endogenous or exogenous rat PT is partially degraded to peptides of these molecular weights by endogenously secreted proteases (15). Further, exogenously added PT was quantitatively recovered by immunoblotting (15, 23). These results demonstrated that highly labeled rat [3H]PT antigen could be efficiently isolated and quantified even though it is less than 1% of the total secreted protein.

![Fig. 1. PT synthesis by hepatoma cells is induced after incubation with bovine PT or rat PT. Cells were incubated for 1.5 hr with exogenous PT added to the culture medium in which the cells had been maintained for 20 hr (non-re-feed protocol). After incubation with [3H]leucine, labeled rat PT was purified by affinity chromatography (see Materials and Methods and refs. 15 and 20) and analyzed by NaDodSO4/polyacrylamide gel electrophoresis. Gel slices were 1 mm; counting efficiencies were 65%. Radioactivity profiles of gel slices derived from the following experiments are shown: A, Control cells (no additions); B, cells incubated with 0.13 μM bovine PT; C, cells incubated with 0.13 μM rat PT. The position of migration of rat PT is indicated ( ).](image)

![Fig. 2. Quantification of the induction of rat PT synthesis by hepatoma cells incubated with exogenous PT. The data are expressed as the percent of total extracellular [3H]leucine-labeled protein that was rat PT as determined by analysis of individual NaDodSO4 gel slices (see Fig. 1). Empty bars, control; hatched bars, cells incubated with 0.13 μM bovine PT; solid bars, cells incubated with 0.13 μM rat PT. (A) Results calculated from the radiochromatograms shown in Fig. 1. (B) Results from identical experiments except that 1 mM benzamidine- HCl was present during the incubation period. Fold increases in rat PT synthesis compared to control levels are shown in parentheses.](image)

Addition of rat PT or bovine PT to the cells under these non-re-feed conditions resulted in significant increases in the quantities of labeled secreted rat PT and its proteolytic byproducts (Figs. 1 and 2A). When expressed as the percent of total extracellular [3H]-labeled protein (Fig. 2A), newly synthesized rat PT increased 2- and 3-fold in hepatoma cells exposed to exogenous bovine PT and rat PT, respectively. On the other hand, identical experiments conducted in the presence of the protease inhibitor benzamidine-HCl (Fig. 2B) revealed minimal differences in the rates of rat PT synthesis. In the presence of benzamidine, exogenously added PT was degraded less than 15%, whereas in the absence of the inhibitor it was degraded more than 80% (results not shown). These results suggested that a proteolytic fragment derived from PT induced synthesis of rat PT. Under conditions in which proteolysis of exogenous PT was prevented, significant increases in secreted rat PT were not observed.

**PT Fragment 1 (Residues 1-156) Induces PT Synthesis.** The data presented in Figs. 1 and 2 showed that the cells could be induced to synthesize more PT only if exogenously added PT underwent partial proteolytic degradation. The ability of purified bovine PT fragment 1 to induce PT synthesis was, therefore, investigated (Table 1). Bovine PT fragment 1 (0.26 μM)

| Table 1. Induction of rat PT synthesis by bovine PT fragment 1 is enhanced by simultaneous administration of a protease inhibitor and re-feeding |
|---|---|---|---|
| Exogenously added polypeptide* | Rat [3H]PT/ extracellular [3H]-protein, %† | Non-re-feed | Re-feed | Double-re-feed |
| None | 0.24 | 0.17 | 0.13 |
| Bovine PT fragment 1 | 0.35 (1.46) | 0.44 (2.59) | 0.53 (4.08) |
| None + benzamidine-HCl | 0.21 | 0.14 | 0.08‡ |
| Bovine PT fragment 1 + benzamidine-HCl | 0.57 (2.71) | 0.42 (3.00) | 0.39 (4.87) |

* Incubation was for 1 hr. Bovine PT fragment 1 was 0.26 μM when present; benzamidine-HCl was 1 mM.
† Data are expressed as pmol of rat [3H]PT/dpm of extracellular [3H]-labeled protein) × 100. See text for full description of cell culture conditions. Fold increases over control levels are shown in parentheses.
‡ Benzamidine-HCl was present during both re-feed cycles.
was added to the medium in which cells had been maintained for 18 hr (non-re-feed conditions). Rat PT synthesis increased by 1.5-fold over the control (no polypeptide addition). However, when benzamidine was added with bovine PT fragment 1, the increase in PT synthesis was 2.7-fold. This result demonstrated that residues 1–156 of PT contained the structural elements required for induction and that inhibition of endogenously secreted, benzamidine-sensitive proteases enhanced the induction.

To study more extensively the effects of the endogenous proteases on the induction, culture conditions were varied (Table 1). Cells maintained for 18 hr in serum-free medium were then re-fed with either fresh medium alone or fresh medium containing bovine PT fragment 1 for 1 hr (re-feed protocol). Under these conditions, bovine PT fragment 1 induced PT synthesis by 2.6- to 3.0-fold, depending upon whether benzamidine was present. The control levels of rat PT synthesis were decreased under these re-feed conditions (compare Table 1, re-feed with non-re-feed). These results indicated that reduction of protease activity by re-feeding with fresh medium allowed greater induction of PT synthesis.

Endogenous proteolysis was further reduced by using a double-re-feed protocol to study induction (Table 1). Cells maintained for 18 hr in serum-free medium were incubated for 1 hr with fresh medium and then re-fed a second time with fresh medium with or without bovine PT fragment 1. Under these conditions, the addition of bovine fragment 1 resulted in 4- to 5-fold increases in rat PT synthesis, while the decrease in the rate of control PT synthesis was greatest after exposure of the system to benzamidine. Additional experiments indicated that benzamidine did not significantly alter total protein synthesis and secretion in non-re-feed and re-feed experiments; however, its repeated administration in double-re-feed experiments resulted in a 40% decrease in total extracellular 3H-labeled protein (results not shown).

These experiments demonstrated that (i) bovine PT fragment 1 induced PT synthesis; (ii) proteolytic activation of fragment 1 was not required for induction; and (iii) removal of proteases by re-feeding reduced control levels of PT synthesis and produced dramatic increases in the induction of PT synthesis (up to 5-fold).

**Bovine PT Fragment 1 Induction Is Dose Dependent.** Cells were incubated with increasing concentrations of bovine PT fragment 1. In this, and all other experiments discussed below, cells were maintained for 18 hr in serum-free medium and then re-fed with fresh medium containing the various polypeptides derived from PT (re-feed protocol) in order to minimize effects of endogenous proteases. The results (Fig. 3) indicated that the bovine PT fragment 1-mediated induction of rat PT synthesis occurred in a dose-dependent manner, reaching apparent saturation between 0.26 and 0.52 μM.

**Identification of the Structural Determinants Required for Induction.** Bovine PT fragment 1 induced PT synthesis by these cells (see above and Table 2). Control experiments using other fragments of bovine PT (see Fig. 4 for location in PT) were performed. Bovine thrombin (residues 275–582) and fragment 2 (residues 156–274) did not stimulate PT synthesis (Table 2). The fragment 1 portion of bovine PT possesses two structural features that distinguish it from the remainder of the molecule. It contains 10 γ-carboxyglutamate residues located near the NH2 terminus and two asparagine-linked oligosaccharide chains at residues 77 and 101 (Fig. 4). To evaluate the inductive capacity of the two structural domains, bovine PT fragment 1 was treated with chymotrypsin (18) to yield the γ-carboxyglutamyl-containing peptide 1–42 and the carbohydrate-containing peptide 46–156. Significant induction of rat PT synthesis was observed only after incubation of cells with the γ-carboxyglutamyl peptide (1–42) (Table 2). Maximal stimulation required a 1- to 2-hr incubation period in the presence of fresh medium. These results indicated that the structural element(s) required for induction resided within the first 42 amino acid residues of PT.

To determine if the γ-carboxyl groups were required for induction, bovine PT fragment 1 was lyophilized in either 0.05 M HCl or 0.01 M KOH and heated at 110°C for 3 hr. As determined from the data presented (Table 2), fragment 1 was expressed in the culture system for 1 hr. The quantity of rat [3H]PT was determined as described for Figs. 1 and 2.

### Table 2. Rat PT synthesis is induced by the γ-carboxyglutamyl peptide of bovine PT (residues 1–42)

<table>
<thead>
<tr>
<th>Exogenously added polypeptide*</th>
<th>Rat [3H]PT/ extracellular ³H-protein, %†</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.15</td>
<td>—</td>
</tr>
<tr>
<td>Thrombin</td>
<td>0.18</td>
<td>1.21</td>
</tr>
<tr>
<td>PT fragment 2</td>
<td>0.17</td>
<td>1.13</td>
</tr>
<tr>
<td>PT fragment 1</td>
<td>0.32</td>
<td>2.13</td>
</tr>
<tr>
<td>Glycopeptide</td>
<td>0.18</td>
<td>1.21</td>
</tr>
<tr>
<td>γ-Carboxyglutamyl peptide</td>
<td>0.34</td>
<td>2.27</td>
</tr>
</tbody>
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* Incubation was for 1 hr. Each polypeptide was 0.13 μM.
† Data are expressed as described for Table 1. Values are representative of between two and eight experiments.

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**Fig. 3.** Bovine fragment 1-mediated induction of rat PT synthesis. Increasing concentrations of fragment 1 were incubated in the culture system for 1 hr. The quantity of rat [3H]PT was determined as described for Figs. 1 and 2.

**Fig. 4.** Diagram of the structure of bovine PT. Gla peptide, the γ-carboxyglutamyl-containing chymotryptic (Ct) peptide 1–42; glyco peptide, the carbohydrate-containing Ct peptide 46–156; Th, site at which thrombin cleaves PT; Xa, sites at which activated factor X cleaves PT; F, positions of asparaginyl-linked oligosaccharide moieties; V, positions of γ-carboxyglutamate residues; fragment 1, the thrombin-releasable NH2-terminal portion of PT (residues 1–156); fragment 2, the thrombin and Factor Xa-releasable portion of PT (residues 157–274); thrombin region, the Xa-releasable thrombin portion of PT (residues 275–582).
termed by amino acid analysis, 95% of the γ-carboxyglutamate residues were converted to glumatic acid after drying from acidic solution, whereas only 15% were converted after drying from basic solution (data not shown). Experiments conducted with these derivatives revealed a 1.2-fold stimulation with the acid-treated peptide, compared to a 1.8- and 2.2-fold induction with base-treated and untreated fragment 1 preparations, respectively (Table 3). That heat treatment did not result in other structural changes in the peptide is supported by (i) amino acid analysis (see Materials and Methods), (ii) the unusual thermal stability of dry proteins (19), and (iii) the relatively high inductive activity of the base-treated species. Because experiments using an equivalent molar dose of free γ-carboxyglutamate did not show any stimulation, these findings indicated that peptide-bound γ-carboxyglutamate residues were required for induction of PT synthesis.

**DISCUSSION**

The ability of the H-35 rat hepatoma cell line to synthesize and secrete biologically active PT in response to vitamin K has been documented (11, 15). The results of Munns et al. (11) indicated that after exposure of H-35 cells to vitamin K for 18 hr, the extracellular PT pool was expanded 2-fold. Because of the latency of this response, it was proposed that an additional control mechanism existed in which the accumulation of vitamin K-dependent by-products resulted in subsequent increases in PT synthesis. The data presented here support this interpretation and provide definitive evidence for such an induction phenomenon. Specific conclusions include: (i) intact PT does not induce its subsequent synthesis; (ii) proteolytic enzymes secreted by the hepatoma cells degrade extracellular PT into peptide fragments that induce PT synthesis; (iii) the structural elements required for induction reside in the NH₂-terminal, γ-carboxyglutamyl-containing portion of PT; (iv) induction by bovine PT fragment 1 is dose dependent; and (v) restricting the accumulation of proteases by successive replacement of the culture medium or by inhibiting protease activity with benzamidine enhances the stimulatory effect of bovine PT fragment 1.

The mechanism by which peptide-bound γ-carboxyglutamate residues induce PT synthesis is uncertain. The regulatory determinants might be the γ-carboxy groups themselves. Alternatively, their presence may impart a particular conformation required for induction. The γ-carboxyglutamate residues probably also confer resistance of the inductive domain to proteolytic degradation (12, 24). That peptide 1–42 itself was sufficient for stimulation indicates that the inductive elements are confined to a limited region.

Although we found that exogenous bovine PT was only half as active as rat PT in stimulating endogenous rat PT synthesis (Figs. 1 and 2), bovine PT fragments were chosen as the source of inducing agents because of their availability in quantity in purified form and their common structural features with rat PT—e.g., similar molecular weights and carbohydrate and amino acid compositions (14). Moreover, the entire amino acid sequence of bovine PT has been determined, whereas that of rat PT is incomplete (12, 14). Thus, while the inductive activity of the corresponding rat PT fragments remains to be determined, our results have identified the structural determinants required for induction.

Our previous data have shown that vitamin K administration to H-35 cells results in an increased rate of PT processing and secretion, and an accumulation of extracellular PT without any alteration in total protein synthesis (11, 15, 20). Vitamin K was present, therefore, for the duration of each experiment to ensure optimal secretion of mature PT. While incubation of cells with exogenous bovine PT or its fragments was characterized by 2- to 5-fold increases in the quantity of endogenously secreted PT, total protein synthesis and secretion remained unchanged. Because the intracellular levels of PT never increased more than 50–70% (data not shown), we conclude that increases in extracellular rat PT induced by bovine PT fragments are due to increases in the synthetic rate of PT and not to changes in secretion rate or alterations in overall protein synthesis.

The mechanism by which γ-carboxyglutamyl-containing PT fragments stimulate PT synthesis remains unknown. Because the synthesis by bovine PT fragment 1 attained apparent saturation (Fig. 3), a cell surface receptor-mediated process may be involved. Such an interpretation would explain why benzamidine administration and re-feeding caused increases in bovine PT fragment 1 stimulatory activity and decreases in PT synthesis in control experiments (Table 1). As a consequence of successive medium changes and benzamidine-dependent inhibition of endogenous PT proteolysis, the levels of active secreted proteases and regulatory fragments derived from endogenous PT may be progressively depleted. While the increase in bovine PT fragment 1-dependent stimulation could be attributable to the longer half-life of the peptide, the decrease in PT control values may reflect a reduction in the quantity of receptor-bound endogenous fragment. Hence, the increased availability of unoccupied receptors could result in the apparent increase in the ability of exogenously added PT fragments to induce PT synthesis.

The increases in PT synthesis and secretion after incubation with PT fragments are probably due to changes in transcription of the PT gene, because the peptide-dependent induction is blocked by actinomycin D (unpublished observations). However, we have not investigated whether alterations in processing of PT precursor mRNA or in the rate of translation of the mRNA might also occur.

In conclusion, we have demonstrated that the de novo synthesis of PT in H-35 cells is regulated by γ-carboxyglutamyl-containing proteolytic fragments of PT itself. Thus we have discovered that a polypeptide derived from a parent protein can subsequently function to induce the synthesis of that protein. This induction may represent a physiologic mechanism for maintenance of plasma PT levels, because the levels of PT fragment 1 that induced synthesis (0.13 μM) might well circulate in vivo (plasma PT is present at 1.6 μM and has a half-life of 7 hr). Coagulation factors VII, IX, and X share extensive sequence homology in their NH₂-terminal regions with PT (2). It will be of interest to determine whether synthesis of these factors is induced by PT fragments as well. Alternatively, breakdown products of other clotting factors might function specifically to feedback-induce their synthesis.
The authors express their appreciation to Dr. Van R. Potter and Ms. Joyce Becker for provision of initial stocks of H-35 rat hepatoma cells. We thank Drs. C. Jackson and L. Glaser for helpful discussions and critical review of the manuscript. The assistance of Ms. S. Winkler, Mrs. S. Silverman, and Mrs. S. Frensky is gratefully acknowledged. This work was supported by grants from the National Institutes of Health (AM 20407 to A.W.S. and HL 12820 to C. Jackson). A.W.S. is an Established Investigator of the American Heart Association, and T.L.C. is a Missouri Affiliate Fellow of the American Heart Association.