Purification, Characterization, and cDNA Cloning of a Kunitz-type Proteinase Inhibitor Secreted by the Porcine Uterus*

(Received for publication, May 9, 1994, and in revised form, July 25, 1994)

Melody L. Stallings-Mann†, Michael G. Burke§, William E. Trout†, and R. Michael Roberts††

From the Departments of †Animal Sciences and §Biochemistry, University of Missouri, Columbia, Missouri 65211

The porcine uterus synthesizes a proteinase inhibitor (M, 14,000) under the influence of progesterone that is relatively specific for plasmin and trypsin. Several isoforms of this uterine plasmin/trypsin inhibitor were purified by a procedure whose final two steps involved affinity chromatography on immobilized chymotrypsin and cation exchange chromatography. Amino-terminal sequencing showed that at least three of the isoforms were closely related. An oligonucleotide probe based on the protein sequence was used to identify cDNA that contained an open reading frame coding for a mature protein (M, 10,295) of 93 amino acids. The inhibitor had a well defined, but unique, Kunitz domain of 64 residues at its amino terminus that shared 67% sequence identity to bovine pancreatic trypsin inhibitor. Its P1 residue was arginine rather than lysine. Northern analysis showed the presence of a single mRNA species (700 bases) that in adult female pigs appeared to be confined to the uterus. During pregnancy, UPTI mRNA expression was high until Day 30 and decreased significantly thereafter. By contrast, uteroferrin mRNA reached maximal concentrations in late pregnancy. These data are consistent with an earlier hypothesis that the inhibitor serves to neutralize the activities of one or more serine proteinases generated by the proliferating trophoblast during the formation of the noninvasive placenta of the pig.

In most animals, implantation involves intrusion of the trophoblast into the uterine wall, thus bringing the developing placenta into close proximity of maternal blood vessels, a process that facilitates the transfer of nutrients from the dam to the fetus. Implantation has been associated with proteinase secretion (6, 7), the failure of the pig blastocyst to invade the uterine wall, despite production of proteinases, raised the possibility that the maternal uterus might secrete proteinase inhibitors. Such inhibitory activity was subsequently identified in the uterine secretions of pigs during pregnancy (11, 14). The activity could be attributed to a group of basic, low molecular weight proteins and was directed toward plasmin, trypsin, and, to a lesser extent, chymotrypsin (18). Further characterization of the uterine plasmin/trypsin inhibitor (UPTI) indicated its presence in uterine secretions of pigs during the luteal phase (11) and pseudopregnancy (15), i.e., when levels of progesterone were high. Plasmin/trypsin inhibitory activity could also be detected in the uterine flushes of ovarioctomized gilts treated with either progesterone or progesterone and estrogen in combination but not with estrogen alone (11, 15, 16). UPTI was identified immunocytochemically in the surface and glandular epithelium of the endometrium (16), and its initial secretion appeared to be triggered by the release of estrogen from conceptuses as they elongated from the spherical to the filamentous form between Days 11 and 13 of pregnancy (14). The aims of the present study have been to purify UPTI, to clone its cDNA, and to examine its expression in the uterus during pregnancy.

EXPERIMENTAL PROCEDURES

Purification of UPTI—Uterine flushings obtained as described previously (17) were centrifuged at 5,800 × g for 45 min at 4 °C, and the supernatant fraction was dialyzed (4 °C) against 0.01 M Tris-HCl (pH 8.2). Basic proteins were enriched by allowing them to bind to CM-cellulose at pH 8.2 in 0.01 M Tris-HCl buffer. They were then eluted in the same buffer containing 0.5 M NaCl. These basic proteins were then size-fractionated by gel filtration over a Sephadex G-100 column (3.5 × 100 cm, Pharmacia Biotech Inc.), and the low molecular weight fraction (range of M, 10,000 to 20,000) was pooled, dialyzed against Tris-HCl buffer (0.01 M, pH 8.2), and loaded onto either an anhydro-chymotrypsin-Sepharose column (see below) or a similar column carrying bound chymotrypsin. After washing with 0.2 M NaCl, bound proteins were eluted with 0.1 M glycine, 0.15 M NaCl (pH 2.4). Fractions (1 ml) were neutralized by addition of 0.2 ml Tris-HCl (1.0 M, pH 8.0). Pooled protein was further fractionated into different charge forms on a Mono-S cation exchange column (0.7 × 5.0 cm, Pharmacia). Protein composition of fractions was assayed by SDS-polyacrylamide gel electrophoresis (18, 19).

Preparation of Anhydro-chymotrypsin and Chymotrypsin Affinity Matrices—Anhydro-chymotrypsin was prepared according to the procedure of Matta et al. (20) and affinity-purified over a luna bean inhibitor-Sepharose column, which was prepared as described previously (21).
Porcine Uterine Proteinase Inhibitor

The resulting material was dialyzed against 0.01 M NaHCO₃ (pH 8.3) and coupled to CNBr-activated Sepharose (1 mg of protein/g of gel, Sigma).

In subsequent experiments, it was noted that chymotrypsin (type VII, 1-chloro-3-tosylamido-7-amino-2-heptanone-treated; Sigma) could substitute as an effective affinity substrate for anhydro-chymotrypsin and coupled to CNBr-activated Sepharose in the same manner as used for anhydro-chymotrypsin.

**Trypsin Inhibition Assay—**Samples (0–100 µl) were incubated with 100 µl trypsin (20 µg/ml, type XIII, i-tosylamide-2-phenylthyl chro- mophore, Sigma) at room temperature for 15 min. The volume was adjusted to 450 µl by addition of 0.05 M Hepes (pH 7.0) and 50 µl of 50 mM N-benzoyl-DL-arginine-p-nitroanilide (stored as a 50 mM stock in dimethyl sulfoxide, Sigma) were added. After incubating 15 min at 37 °C, 500 µl of soybean trypsin inhibitor (10 µg/ml, type I-S, Sigma) were added. Absorbance at 410 nm was measured. Since UPTI had previously been shown to bind trypsin very tightly in a 1:1 stoichiometry (15), the amount of functional UPTI in any sample could be measured accurately.

**Amino Acid Sequencing—**UPTI was subjected to NH₂-terminal amino acid sequence analysis by the Edman degradation method on an Applied Biosystems model 470 protein sequencer with on-line analysis for phenylthiohydantoin derivatives (Protein Core, University of Missouri, Columbia, MO).

**Design of Oligonucleotide for Library Screen—**NH₂-terminal amino acid sequencing of UPTI indicated considerable similarity to porcine leukocyte inhibitor, bovine pancreatic trypsin inhibitor, and porcine serum trypsin inhibitor (23). A region of UPTI highly conserved in these other proteins (amino acids 16–27, see Fig. 2) was chosen, and an oligonucleotide, UPTI-5, was made corresponding to this region. The putative nucleotide sequence, 5'-GGC CCC TGC (data not shown), was then used to screen for muscle, perinephric fat, and kidney was kindly provided by the laboratory of Drs. T. G. Ramsay and M. White (Ohio State University).

**Isolation of RNA from Porcine Tissues and Northern Analysis—**RNA was isolated from porcine tissues as described previously (32). For Northern blot analysis, total cellular RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to nylon filters (Magna NT, Micron Separations Inc., Westboro, MA) according to the method of Sambrook et al. (22). The filters were hybridized in a buffer containing the 32P-labeled UPTI-31 and 32P-labeled uteroferrin cDNA probes as described previously (33).

**Statistical Analysis—**The intensity of autoradiographic signals obtained from Northern blots was determined by densitometry with a Bio-Rad model 620 video densitometer (Bio-Rad). Signal intensity is expressed as the height in optical density units. The differences in intensity of signal for UPTI and uteroferrin were examined by one-way analysis of variance performed by the General Linear Model procedure of the statistical analysis system (SAS Institute, Cary, NC).

**RESULTS**

**Purification of UPTI—**Chromatography of CM-cellulose-positive material over a Sephadex G-100 column yielded five major peaks (I–V) of protein (15, 34) (data not shown). Fraction V contained a mixture of low molecular weight, basic proteins, including the majority of the trypsin inhibitory activity (15). Pooled Fraction V was loaded onto the anhydro-chymotrypsin affinity column, and the trypsin inhibitor, which bound quantitatively, was eluted with a glycine buffer, pH 2.4. Electro- photic analysis of the eluted protein material revealed a single broad band on one-dimensional SDS-polyacrylamide gel electrophoresis gels (data not shown). Treatment of sample with β-mercaptoethanol just prior to electrophoresis did not result in a change in migration of the band representing UPTI. Fig. 1 shows the elution profile of the affinity-purified fraction during chromatography on a Mono S FPLC column (Pharmacia). The gradient was linear (0.00–0.43 M NaCl; 60 ml; 60 min). Polypeptides were detected by continuous monitoring of the column effluent at 280 nm (open circles). Each fraction was assayed for UPTI activity in a trypsin inhibition assay. The percent inhibition of a trypsin standard is shown (closed circles).

**Amino Acid Sequencing—**Samples from both the first and last peaks (Fractions 17 and 23) that eluted from the Mono S ion exchange column (Fig. 1) were subjected to NH₂-terminal amino acid sequence analysis. Sequence information was obtained for the first 24 residues from Fraction 17 and the first 29 residues from Fraction 23 (Fig. 2). Fraction 17 yielded two
signals, a minor one, starting with a valine residue followed by arginine, and a major signal starting with an alanine. These two signals were clearly variants of the same sequence, with the minor component lacking the two terminal residues found on the minor component. Fraction 23 gave a single sequence identical to the minor signal present in Fraction 17.

The NH₂-terminal residue of UPTI was similar to the NH₂ terminus of native bovine pancreatic trypsin inhibitor and porcine leukocyte inhibitor (Fig. 2). The degree of sequence identity in the region of overlap was 66.7% for bovine pancreatic trypsin inhibitor and 75.0% for porcine leukocyte inhibitor. A key difference between UPTI and the other two inhibitors was the putative active site residue, P₁ (35). UPTI contained an arginine residue, whereas bovine pancreatic trypsin inhibitor (36) and porcine leukocyte inhibitor (22) contained a lysine residue at P₁. The putative active site residue, P₁, is indicated by an arrow. BPTI, bovine pancreatic trypsin inhibitor (36); PLI, porcine leukocyte inhibitor (24).

**Fig. 2.** NH₂-terminal amino acid sequences of UPTI isoforms compared to bovine pancreatic trypsin inhibitor and porcine leukocyte inhibitor. The single-letter code for amino acids is used throughout. Residues common to UPTI and the other two sequences are boxed. The amino acids used to design the oligonucleotide for library screening are underlined. The putative active site residue, P₁, is indicated by an arrow. BPTI, bovine pancreatic trypsin inhibitor (36); PLI, porcine leukocyte inhibitor (24).

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Cloning and Sequencing of UPTI cDNA—A 17-base oligonucleotide probe was designed to represent a region of UPTI, amino acids 16–27 (Fig. 2), which is highly conserved in other Kunitz-domain proteins such as bovine pancreatic trypsin inhibitor and porcine leukocyte inhibitor. The putative nucleotide sequence was derived by assuming that amino acid differences between the porcine leukocyte inhibitor (22) and UPTI arose as the result of point mutations and by examining frequencies of codon usage in the pig (24). This probe was used to screen approximately 100,000 plaques of a porcine endometrial cDNA library from Day 60 of pregnancy (27). Only 7 positive plaques were clearly variants of the same sequence, with two signals being variants of the same sequence, with the minor component lacking the two terminal residues found on the minor component. Fraction 23 gave a single sequence identical to the minor signal present in Fraction 17.

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**Fig. 3.** The cDNA sequence for UPTI and the inferred sequence of the protein. The nucleotide sequence is displayed above the inferred amino acid sequence. Encoded amino acids are represented by their single-letter code under the appropriate codon. The 4 additional bases present in UPTI-1 (positions 15–18) are indicated in boldface type. The signal peptide is underscored with a solid line. The polyadenylation signal is indicated by asterisks. The boxed sequence indicates the extent of the UPTI-31 probe used for the Northern analyses.

**DISCUSSION**

UPTI consists of a group of basic, low molecular weight proteins secreted by the porcine uterus under the influence of progesterone (11, 14–16), which has inhibitory activity toward plasmin, trypsin, and, to some extent, chymotrypsin (15). The purification procedure described in this paper separates several of these forms, three of which were subjected to NH₂-terminal amino acid sequencing. The sequences obtained were Day 60 library. Therefore, there was no evidence that the protein isoforms arose from distinct transcripts. The complete open reading frame of UPTI-1 started at position 15 and coded for a 122-amino acid polypeptide. The inferred signal peptide is indicated by a solid line. The polyadenylation signal is indicated by asterisks. The boxed sequence indicates the extent of the UPTI-31 probe used for the Northern analyses.

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Porcine Uterine Proteinase Inhibitor

Day bands and the endometrium hybridization signal for both probes could be detected in all stages of individual animals at each time point. The blot was probed with a UPTI cDNA sequenced were identical in the regions where they two amino acids, Val and Arg, in one form. In addition, all four

Kunitz domains (38). Kunitz inhibitors form unusually stable enzyme-inhibitor complexes, due to the ability of the inhibitor to act as a very effective substrate analog (39). Two binding loops define their specificities and form interfaces with the proteinase target (40). The residue located at the center of the first loop, the P1 residue, mimics bound peptide substrate and defines the primary specificity toward particular classes of proteinases (41). The P1 residue is most commonly occupied by a lysine. Additional specificity is conferred by the residues on each end of the binding loops, which interact with proteinase atoms around the perimeter of the catalytic site (42). UPTI has many of the characteristics of a classic Kunitz-type inhibitor, including its low molecular weight and basic isoelectric point, its single inhibitory domain located at the NH2 terminus, and strict conservation of its 6 cysteine residues. By comparison with bovine pancreatic trypsin inhibitor, with which it shares 66.7% sequence identity, the two proteinase-binding loops are most likely located at residues 15–23 and 38–45 in UPTI (41). The putative P1 residue is occupied by arginine, a conservative replacement for lysine. Both residues are common for those Kunitz inhibitors that cleave at basic residues (36, 42). Differences among the other residues in the binding loops between UPTI and other Kunitz inhibitors likely contribute to more subtle changes in inhibitor specificity among these proteins (41).

Bovine pancreatic trypsin inhibitor is synthesized as a larger precursor, containing both a signal peptide and a pro-region (42). Sequence analysis of the cDNA for UPTI strongly suggests that it must also be proteolytically processed. The pre-region has the characteristics of a signal peptide (43), but the site of signal peptide cleavage is difficult to predict (43, 44), and the length of the pro-region (if one indeed exists) is unclear. Most likely cleavage occurs between Ser20 and Thr21, since this site conforms more closely than any other to the (−3,−1) rule, in which the residue at −3 is not aromatic, charged, or bulky, and the residue at −1 is small (44). The remaining 9 amino acids would then constitute a short pro-region. Significantly, UPTI does not have a cysteine residue in a position analogous to that found in the pro-region of bovine pancreatic trypsin inhibitor. Such a cysteine has been suggested to have a crucial role in folding of the Kunitz domain (45). Its absence in UPTI suggests that the requirement for a pro-region cysteine is not universal among Kunitz inhibitors.

The synthesis of UPTI is strongly under the influence of progesterone (15). Amounts produced, therefore, are high during the luteal phase of the estrous cycle (11, 16), as well as during pregnancy (14) and pseudopregnancy (15). Our results from the Northern analyses are consistent with this hypothesis, since expression of the UPTI mRNA was detected during all stages of pregnancy examined. However, expression was clearly stronger at Day 30 of the 114-day gestation period, a time at which rapid expansion and development of the allantois is occurring (46). This decrease in expression of the UPTI mRNA during later pregnancy is in contrast to uteroferrin (Fig. 5; Ref. 26), the uterine serpin (47), and retinol-binding protein (48), whose mRNA expression remain high. Thus, it seems likely that UPTI functions mainly in early pregnancy, at which time it may, as previously suggested, help to control proteolytic cascades initiated by the potentially invasive pig trophoblast (11, 14–16).

Acknowledgments—We thank August Rieke for organizing the breeding and transport of animals, Dr. Craig Jones and Dana Thomas for surgical assistance, Bosena Szafirowska and Jodie Duffy for help with collection of tissue from slaughtered gilts, Dr. Joe Forrester for synthesis of oligonucleotides, Dr. Sam Araghi for protein sequencing, and Gail Foristal for preparing the manuscript.
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