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Isolation, characterization and partial sequencing of Pregnancy Associated Mouse Protein PAMP1 identifies it as a novel female specific protein, unrelated to the α-2-macroglobulin family of proteinase inhibitors

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Pregnancy Associated Mouse Protein 1 (PAMP1) was isolated from plasma of female mice. An antiserum raised against the purified protein confirmed its immunochemical identity with the originally described PAMP1. Pregnant females were observed to have plasma levels of PAMP1 that are increased two-fold at day 10–13 of gestation relative to non-pregnant females, while male mouse plasma did not contain PAMP1. The purified protein displayed an apparent subunit molecular mass of 70 kDa, irrespective of cystine reduction. The native molecular mass, estimated by gel-filtration, was about 140 kDa, indicating that PAMP1 is circulating as a non-covalent homodimer. The amino-terminal sequence of the intact protein and the internal sequences of four cyanogen bromide fragments demonstrated that this protein is not related to any known member of the α-2-macroglobulin family nor to any protein in the sequence databases. The physicochemical and the sequence data thus establish this protein as a novel, female-specific protein, but unrelated to the Macroglobulin proteinase inhibitor family.

Pregnancy protein; α-2-Macroglobulin

1. INTRODUCTION

Pregnancy-associated proteins have been intensely studied, both for clinical and for fundamental reasons. One of these proteins, Pregnancy Associated Mouse Protein 1 or PAMP1, was shown by immunochemical means to be a possible murine counterpart of the human Pregnancy Zone Protein (PZP) [1–4]. PZP is a proteinase inhibitor and scavenger of the α-2-macroglobulin (A2M) family [5–7]. Expression of PZP is normally restricted to pregnant women with highest blood levels in the third trimester of pregnancy [2,8,9]. The role of PZP in vivo, its precise target proteinases or other scavenger functions, remains enigmatic in view of the already abundant presence of A2M, the constitutional wide-spectrum proteinase scavenger in humans [7,10]. Since neither the site of synthesis of PZP nor the factors regulating its synthesis are known and since no human cell lines secreting PZP are available, an animal model system would be an important alternative to the human system.

Since we are engaged in the characterization of the murine A2M family of proteinase inhibitors and their receptor [11–16] we became interested in PAMP1 as a possible PZP homolog. We report here the isolation of PAMP1 from female mouse plasma and the results of the analysis of its main structural and physical characteristics. The findings demonstrate that this protein is structurally and functionally not related to A2M or PZP. The N-terminal and internal protein sequences obtained indicate it to be an unknown protein showing no sequence identity with any known protein. Moreover, its presence in the plasma of female mice irrespective of their gestational status and the virtual absence of PAMP1 in the plasma of all male mice examined, leaves us to conclude that PAMP1 is a female-specific but not a pregnancy-associated protein.

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Abbreviations: A2M, α-2-macroglobulin; PZP, pregnancy zone protein; PAMP, pregnancy associated mouse protein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

2. MATERIALS AND METHODS

2.1. *Isolation of PAMP1 from mouse plasma*

Mice from the NMRI strain were used throughout. Mouse blood was collected from anesthesized animals by cardiac puncture in heparinized syringues. After centrifugation the plasma (5 ml) was treated with polyethyleneglycol 6000 (5% final concentration). After mixing for 30 min at 4°C, the clear supernatant, collected after centrifugation (8,000 × *g*, 30 min), was subjected to gelfiltration on a column (2.6 × 95 cm) containing ACA 34 (LKB-Pharmacia) and developed in phos-

phate-buffered saline (PBS, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4). The peak corresponding to PAMP1 was identified by rocket immunoelectrophoresis and fractions were pooled and concentrated. Subsequently, hydrophobic interaction chromatography on a TSK Phenyl-5PW column [17] was done to separate PAMP1 from Murinoglobulin, albumin and immunoglobulins. The final purification step consisted of high performance gelfiltration on columns (1 × 30 cm) of Superose-6 and Superose-12 (Pharmacia) coupled in series. Column fractions and pools were analyzed by SDS-PAGE and by rocket immunoelectrophoresis.

Original antisera against PAMP1 were prepared by immunization of rabbits or goats with pregnant mouse serum and absorbed with male mouse sera [1,2]. These were used to monitor the purification of PAMP1 from mouse plasma. The purified PAMP1 protein was used to immunize rabbits to obtain monospecific polyclonal antibodies.

Rocket immunoelectrophoresis was carried out on glass plates, in 1% agarose gels in 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4 (TBE) containing the specified antiserum. After electrophoresis (overnight at 2.5 V/cm) the gels were soaked, dried and stained with Coomassie brilliant blue and destained by diffusion.

2.2. *N-terminal protein sequencing*

N-terminal aminoacid microsequencing of intact proteins was done after binding of the protein (about 100 pmol) to Biobrene treated glassfilters. For cyanogen bromide fragmentation, PAMP1 was dissolved in 70% formic acid and treated with CNBr in a 100-fold molar excess for 18 h at room temperature. After lyophilization and resuspending in water (repeated three times), the residue was dissolved in SDS sample buffer and the peptides separated by SDS-PAGE [11,18]. After electrotransfer to Problott membranes, the membranes were stained briefly with Coomassie brillant blue, washed exhaustively in water and the stained bands cut out for direct solid state sequencing according to the procedures of the manufacturer (Applied Biosystems). N-terminal protein microsequencing was performed on an Applied Biosystems Model 477A pulsed liquid phase sequencer with on-line phenylthiohydantion-amino acid identification (Applied Biosystem 120A Analyser).

3. RESULTS

PAMP1 was isolated from heparinized mouse plasma by a simple procedure including polyethylene glycol precipitation, followed by a first gelfiltration step. Subsequently, hydrophobic interaction chromatography (HIC-HPLC) was performed on a phenyl derivatized matrix (Fig. 1). PAMP1 binds to this matrix under conditions (0.1 M sodium phosphate, 0.5 M sodium sulphate, pH 6.5) which are identical to those used for binding of native human A2M and PZP [6,17]. PAMP1 is eluted from the column at the end of the gradient (Fig. 1). Since we used water as the limiting eluent, this indicated that PAMP1 is similar to native A2M and PZP rather hydrophobic in nature. However with all preparations of A2M and PZP (even native protein preparations) analyzed to date, we have observed more complicated chromatograms. This is due to inactivated and complexed forms of A2M and PZP which elute earlier in the gradient [17]. This was never observed with PAMP1, which eluted at the end of the gradient in a single peak.

Further purification was by high performance gelfiltration (Fig. 2). This also allowed us to estimate the native molecular mass of the protein at about 140 kDa

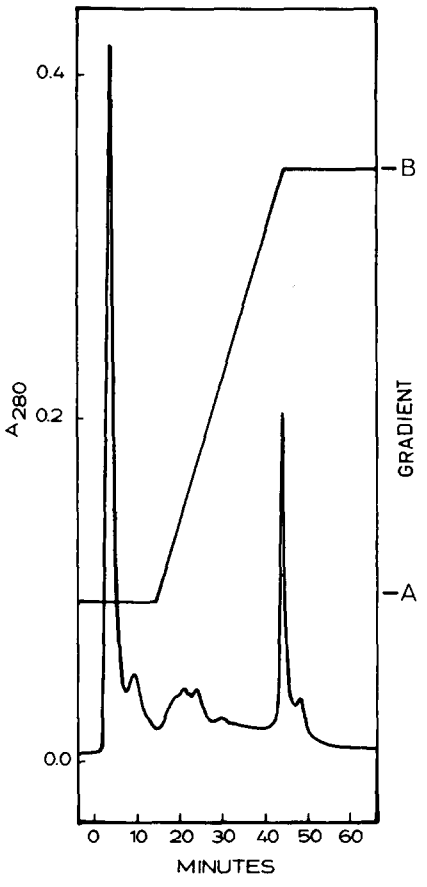

Fig. 1. Hydrophobic interaction chromatography of PAMP1. Fractions containing PAMP1 were applied on a Phenyl TSK-5PW column in 0.1 M sodium phosphate, 0.5 M sodium sulfate, pH 6.5. After washing with the same buffer, a linear gradient was used to develop the column, at a flowrate of 1 ml/min. The limiting eluent was water [17]. Under these conditions PAMP1 is eluted as a sharp peak at the end of the gradient, an elution behaviour which is very similar to that of native α-2-macroglobulin [17]. The absorbance tracing at 280 nm and the linear gradient profile (from 0–100% water) are shown.

(using A2M, immunoglobulin and albumin to calibrate the columns), identical to the reported value [2]. The subunit molecular mass, which to our knowledge has not been determined before, was estimated at 70 kDa under denaturing and reducing conditions (Fig. 3). Without cystine reduction, the apparent molecular weight increased only slightly to about 75 kDa (Fig. 3).

The purified protein was used to immunize rabbits. The antiserum displayed a complete identity in double immunodiffusion tests with an antiserum originally used to demonstrate the existence of PAMP1 [2]. The purified protein did not react with polyclonal antisera against human A2M, human PZP, mouse A2M or mouse Murinoglobulin.

Rocket immunoelectrophoresis of plasma from male and from normal and pregnant female mice showed the presence of PAMP1 in all female sera and its complete absence in plasma from male mice (Fig. 4). Normal

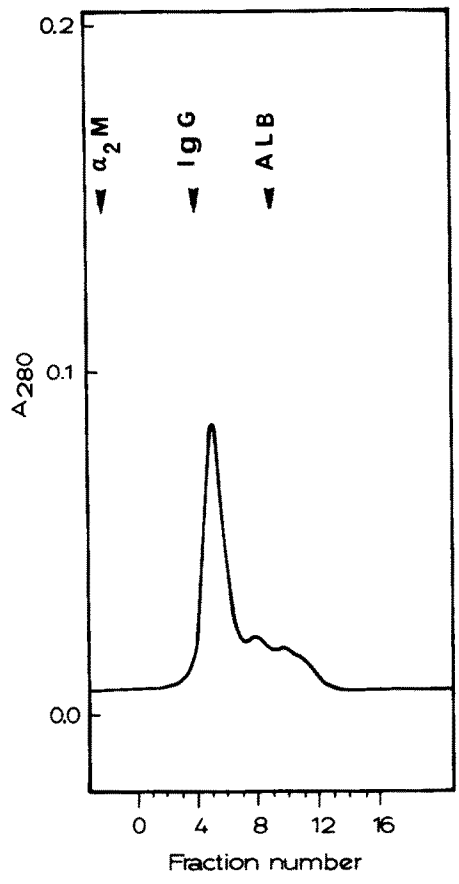

Fig. 2. High performance gelfiltration of PAMP1. Pooled fractions of the Phenyl-5PW column containing PAMP1 were concentrated to 250 μl and applied on columns of Superose-6 and Superose-12 (Pharmacia), coupled in series. Elution was carried out with Tris-buffered saline (10 mM Tris, 0.15 M NaCl, pH 7.6) at a flowrate of 0.75 ml/min and fractions of 3 ml were collected. Fractions 4–6 contained pure PAMP1 and were pooled and concentrated.

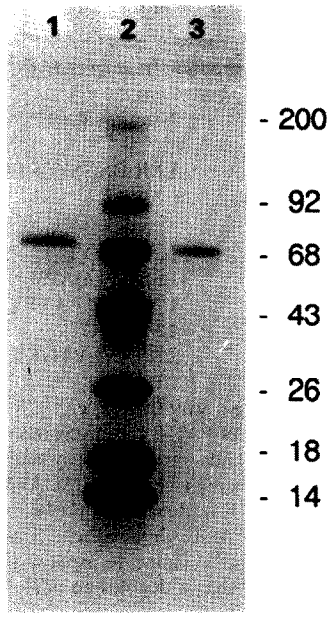

Fig. 3. Subunit molecular mass of PAMP1. The equivalent of 5 μg protein was applied after denaturation with or without reduction (1% SDS in 50 mM Tris, pH 8.0, with or without 1% 2-mercaptoethanol kept at 100°C for 5 min). Lane 1: PAMP1, unreduced. Lane 2: markers with M_r as indicated. Lane 3: PAMP1, reduced.

4. DISCUSSION

We have isolated the mouse protein, previously identified and named PAMP1, in order to examine its relation to the α-2-macroglobulin family of proteinase inhibitors. This relation was originally claimed based on the immunochemical cross-reaction between this protein and the human Pregnancy Zone Protein [1–3].

levels of PAMP1 in female mice were estimated at between 0.2 and 0.5 μg per ml plasma, increasing to about double these values in mid-gestation (day 10–13).

Aminoterminal sequencing of the purified protein yielded a unique sequence of 15 amino acids (Table I). Internal sequences were obtained after partial cyanogen bromide cleavage of the protein, separation of the fragments by SDS-PAGE and electroblotting. A 30 kDa CNBr fragment yielded the same aminoterminal sequence as the intact PAMP1, while the other fragments yielded unique sequences. All the sequences determined were different from N-terminal or internal protein sequences of the members of the A2M family of proteinase inhibitors known to us (human A2M and PZP, mouse A2M and Murinoglobulin, rat A1M, A2M and A1 inhibitor 3). Furthermore, no significant sequence identity was detected with any protein in the protein sequence database (Swissprot) nor, after reverse translation, in the nucleotide sequence databases consulted (EMBL and Genbank).

Fig. 4. Rocket immunoelectrophoresis of PAMP1 in mouse plasma. Mouse plasma (m = male, f = female and pf = pregnant female) was diluted ten-fold in 0.15 M NaCl and 5 μl samples were applied in the wells. The gel contained 7.5% (v/v) of a polyclonal rabbit antiserum directed against PAMP1. A weak cross reaction with an unidentified plasma protein was noted in all samples, also in the male plasma samples.

Table I

Aminoterminal protein sequences of intact PAMP1 and of its cyanogen bromide fragments.

	N-terminal sequence
Intact PAMP1	LMLDSGSEPKLIAEP
CNBr fragments:	
50 kDa	LxPQGPYE
40 kDa	LSDETLQAPV
35 kDa	LNDEGTEAVFEL
30 kDa	LMLDSG
18 kDa	LSNAVEVTGKEPLP

The isolated PAMP1 protein was treated with CNBr and the fragments were separated by SDS-PAGE and electroblotted onto Problott membranes. The peptide bands were located by staining, cut out and used for N-terminal sequencing (see Section 2). Amino acids are denoted by their one letter code (L, leucine; P, proline; Q, glutamine; G, glycine; P, proline; Y, tyrosine; E, glutamic acid; S, serine; D, aspartic acid; T, threonine; A, alanine; V, valine; N, asparagine; F, phenylalanine; M, methionine; K, lysine; x denotes an unidentified residue).

The fysico-chemical characterization of the purified PAMP1 protein yielded a subunit molecular weight of 70 kDa, which was only marginally higher when the protein was analyzed unreduced. This has not been reported previously. The native molecular mass by gelfiltration yielded a value of 140 kDa, similar to the reported value [2]. The N-terminal sequence obtained from the intact protein yielded single derivatives in each cycle, proving that a single species was being analyzed, confirming the single protein band observed in SDS-PAGE. Taken together these results demonstrate that the isolated PAMP1 protein circulates in the murine bloodstream as a 140 kDa, non-covalent homodimer of 70 kDa subunits.

The molecular data and the N-terminal sequences of the isolated protein and of the cyanogen bromide fragments demonstrate conclusively that this protein is not related to the α-2-macroglobulin family of proteinase inhibitors. These sequences are neither related to those of mouse A2M [16] nor to any Murinoglobulin [12], nor to any protein sequence in the sequence databases consulted.

Examination of mouse sera and plasma with the antiserum obtained by immunization with the purified protein confirmed that PAMP1 is restricted to females. Plasma of pregnant females showed the highest concentration around day 10–13 of gestation, with levels about twice normal. Normal levels in females of the NMRI mouse strain were estimated at 0.2–0.5 μg/ml plasma. Male plasma samples were negative when examined by rocket immunoelectrophoresis. The purified protein did not react with specific polyclonal antisera directed against human, A2M or PZP, or against mouse A2M or Murinoglobulin. The original observation regarding the immunochemical crossreaction with antisera against human PZP thereby remains unexplained. Our result do caution however against conclusions based solely on immunochemical findings without support of physico-chemical or sequence data.

We conclude that PAMP1 is not a pregnancy specific but a female specific protein, unrelated to A2M or to any known protein. We propose to rename the protein to FSMP or Female Specific Mouse Protein, until a more appropriate name, referring to the as yet unknown function of the protein, can be coined.

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REFERENCES

[1] Hau, J., Svendsen, P.E., Teisner, B. and Svehag, S. (1978) J. Reprod. Fert. 54, 239–243.
[2] Hau, J., Westergaard, J.G., Svendsen, P., Bach, A. and Teisner, B. (1981) J. Reprod. Immunol. 3, 341–349.
[3] Hau, J., Svendsen, P., Chemnitz, J., Teisner, B. and Westergaard, J.G. (1983) Am. J. Reprod. Immunol. 3, 124–126.
[4] Hau, J. and Porstmann, T. (1984) Lab. Animals 18, 344–348.
[5] Sand, O., Folkersen, J., Westergaard, J.G. and Sottrup-Jensen, L. (1985) J. Biol. Chem. 260, 15723–15735.
[6] Van Leuven, F., Cassiman, J.J. and Van den Berghe, H. (1986) J. Biol. Chem. 261, 16622–16625.
[7] Sottrup-Jensen, L. (1987), The Plasma Proteins, (Putman, F.W., Ed.), Vol. 5, pp. 191–291, Academic Press, Orlando, FL.
[8] Von Schoultz, B. (1974) Am. J. Obstet. Gynecol. 359, 303–310
[9] Folkersen, J., Teisner, B., Grunnet, N., Grudzinskas, J.G., Westergaard, J.G. and Hindersson, P. (1981) Clin. Chim. Acta 110, 139–145.
[10] Van Leuven, F., Cassiman, J.J. and Van den Berghe, H. (1982) Biochem. J. 201, 119–128.
[11] Van Leuven, F., Marynen, P., Cassiman, J.J. and Van den Berghe, H. (1987) J. Biochem. 101, 1181–1189.
[12] Overbergh, L., Torrekens, S., Van Leuven, F. and Van den Berghe, H. (1991) J. Biol. Chem. 266, 16903–16910.
[13] Hilliker, C., Overbergh, L., Petit, P., Van Leuven, F. and Van den Berghe, H. (1992) Mammal. Genome 3, 469–471.
[14] Hilliker, C., Van Leuven, F. and Van den Berghe, H. (1992) Genomics, 13, 472–474.
[15] Van Leuven, F., Torrekens, S., Overbergh, L., Lorent, K., de Strooper, B. and Van den Berghe, H. (1992) Eur. J. Biochem. 210, 319–327.
[16] Van Leuven, F., Stas, L., Raymakers, L., Overbergh, L., de Strooper, B., Hilliker, C., Lorent, K., Fias, E., Umans, L., Torrekens, S., Serneels, L., Moechars, D. and Van den Berghe, H. (1993) Biochim. Biophys. Acta, in press.
[17] Van Leuven, F., Cassiman, J.J. and Van den Berghe, H. (1985) Science Tools 32, 41–43.
[18] Laemmli, U.K. (1970) Nature, 227, 680–685.