Circulating Human Pregnancy-associated Plasma Protein-A Is Disulfide-bridged to the Proform of Eosinophil Major Basic Protein*

(Received for publication, February 26, 1993, and in revised form, April 9, 1993)

Claus Oxvig, Ole Sand, Torsten Kristensen, Gerald J. Gleich, and Lars Sottrup-Jensen

From the Departments of Molecular Biology and Chemistry, University of Aarhus, 8000 Arhus C, Denmark and the Department of Immunology and Medicine, Mayo Clinic and Foundation, Rochester, Minnesota 55905

A previously unrecognized association between pregnancy-associated plasma protein-A (PAPP-A) and the proform of eosinophil major basic protein (proMBP) is demonstrated. PAPP-A isolated from pooled pregnancy serum is shown to be a disulfide-bridged complex with proMBP (PAPP-A/proMBP) in which the subunits of the constituents are present in a 1:1 molar ratio. The results are the outcome of analysis of tryptic and CNBr/tryptic peptides from PAPP-A/proMBP, sequence analysis of intact and reduced and carboxymethylated PAPP-A/proMBP, and reaction with monoclonal antibodies directed against MBP and its proform. In addition, it is shown that commercial polyclonal anti-PAPP-A is polyspecific, also reacting with MBP.

Pregnancy-associated plasma protein-A (PAPP-A)1 is an a2-chain mobile glycoprotein (2-5). It is present at approximately 50 mg/liter in pooled third trimester serum (6, 7), but its physiological role is not known. PAPP-A is believed to consist of two disulfide-bridged 200-250-kDa subunits (2-5). The subunit of PAPP-A in serum contains 1547 amino acid residues and is derived from a large precursor.2 PAPP-A has been localized to the trophoblastic tissue of the placenta (8, 9), which is considered the main source of circulating PAPP-A in pregnancy (8). In nonpregnant individuals PAPP-A has been reported to be present in low amounts in the Graafian follicle and corpus luteum (10, 11), and in the testes and the seminal fluid (12, 13). The major basic protein (MBP) of the eosinophil granule is derived from the 222-residue preproMBP (14-16). In addition to mature MBP (residues 106-222), preproMBP contains a presumed 15- or 16-residue signal peptide and an acidic propiece (residues 16/17-105). MBP isolated from the eosinophil granule is cytotoxic to mammalian cells and has been implicated in tissue damage associated with eosinophil infiltrates (17-19). The plasma level of immunoreactive MBP is greatly elevated in pregnancy, and immunohistochemically, MBP is located to the X-cells and giant cells of placenta (20-22). MBP has been purified from placental tissue (23), and it seems to be tightly associated with unknown large proteins (23, 24).

To initiate cDNA cloning of PAPP-A,2 tryptic peptides of protein isolated from pregnancy serum were characterized. Here we show that several of the peptides in fact are derived from proMBP. We conclude that circulating PAPP-A is a disulfide-bound complex between PAPP-A and proMBP.

EXPERIMENTAL PROCEDURES

Proteins—PAPP-A/proMBP was purified in approximately 60% yield from the supernatant obtained after precipitating pooled pregnancy serum with 16% (w/v) polyethylene glycol 6000 by successive DEAE-Sephacel, N2-chelate, gel chromatography, and negative immunofinity steps.3 MBP was prepared as described earlier (25). Rabbit anti-PAPP-A A230, rabbit anti-complement C9c A062, peroxidase-conjugated rabbit anti-mouse IgG P32, and pig anti-rabbit IgG P217 were from DAKO. Mouse mAb J6-8A4 against MBP was described recently (23). Mouse mAb J163-15E10 against the propeptide of MBP was produced by immunization of mice with proMBP (partially purified from supernatants of Chinese hamster ovary cells transfected with preproMBP cDNA). By immunoblotting J6-8A4 is reactive with proMBP and MBP, whereas J163-15E10 is reactive only with proMBP.4 The mouse mAbs anti-trinitrophenyl (26) and anti-plasminogen activator inhibitor-1 (27) were gifts from P. A. Andreasen. Fetal calf serum was from Life Technologies, Ltd., Scotland.

Materials—Sepharose CL-6B was from Pharmacia. 125I-Labeled oat dextrin was from Amersham. Standard chemicals were from Sigma, Serva, and Merck. PVDF membranes were from Applied Biosystems.

Miscellaneous Procedures—For SDS-PAGE the Tris-glycine system (28) was used (10-20% acrylamide, gel size 0.5 x 80 x 100 mm). Coomassie Brilliant Blue was used for staining. Electrophoretic gel was done for 45 min at 100 V/500 mA using the standard Tris, glycine, 0.1% SDS buffer, pH 8.3. For immunological detection of proteins blotted or spotted onto PVDF, 2% Tween 20 and 1% fetal calf serum were used for blocking. Peroxidase activity was detected by incubation with H2O2 and 3,3'-diaminobenzidine. The cell culture supernatants containing mAbs J6-8A4 and J163-15E10 were diluted 20- and 2000-fold, respectively, and anti-PAPP-A was diluted 2000-fold. Incubations with either of two mAbs of irrelevant specificity (26, 27), with rabbit anti-complement C9c, or without primary antibody were all negative.

Amino Acid and Sequence Analysis—Analysis of amino acids and amino sugars was done by cation exchange (29). Edman degradations of peptides were carried out using Beckman 890C (30) and Applied Biosystems 477A instruments. Peptides (less than 150 pmol) were degraded after being spotted on PVDF.

Preparation of Peptides for Sequence Analysis—Reduced and carboxymethylated PAPP-A/proMBP was digested with 1/50 (w/w) trypsin for 2 h at room temperature. The digest was fractionated by DEAE-Sephacel chromatography using gradient elution with NaH2PO4/NaCl followed by reverse phase HPLC on Nucleosil C18. Fifteen pure peptides were subjected to sequence analysis (TP and TpMBP series, Table I).

* This work was supported by the NOVO Foundation, the Danish Cancer Society, Aarhus University, the Danish Biomembrane Research Center, and National Institutes of Health Grant AI 09728 and AI 11244. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) X68280.

1 To whom correspondence should be addressed: Dept. of Molecular Biology, University of Aarhus, Bldg. 130, 8000 Arhus C, Denmark. Tel.: 45-38125177, ext. 2375.

2 The abbreviations used are: PAPP-A, pregnancy-associated plasma protein-A (human); MBP, eosinophil major basic protein (human); PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; PVDF, polyvinylidene difluoride; mAb, monoclonal antibody; HPLC, high performance liquid chromatography.

3 T. Kristensen, O. Sand, C. Oxvig, N. P. H. Moller, and L. Sottrup-Jensen, manuscript in preparation.

4 P. Popken-Harris, D. A. Loegering, and J. Checkel, unpublished observations.
From a digest aimed at determining the disulfide bridges of PAPP-A/proMBP one peptide (CBT1) connected the PAPP-A and proMBP subunits (Table II). Another peptide (CBT2) spanned the processing site in proMBP (residues 105–106; Refs. 15 and 16). Curiously, that peptide was a dimer containing two disulfide bridges (Table II). The combined results suggested that proMBP is a constituent of circulating PAPP-A. As judged from the average yields of the 12 PAPP-A peptides (22%) and the three proMBP peptides (26%), circulating PAPP-A is a disulfide-bound complex in which the subunits of PAPP-A and proMBP are present in approximately equimolar amounts. PAPP-A/proMBP showed, in addition to the sequence corresponding to the PAPP-A subunit, two NH₂-terminal sequences in an approximately 2:1 molar ratio (Table III) starting at residues 17 and 18 of proproMBP.

\[1^{14}C\text{-Carboxymethylated PAPP-A/proMBP was subjected to denaturing gel chromatography to separate its subunits (Fig. 2). Approximately 84% of the radioactivity associated with protein eluted in fractions 20–29, the remainder in fractions 30–37. Upon SDS-PAGE the material in pool 1 had the expected size of the PAPP-A subunit (not shown), whereas the material in pool 2 (Fig. 1A, lane 4) migrated as a smear corresponding to species of approximately 50–90 kDa rather than 23.4 kDa expected for proMBP (15, 16). The pool 2 material evidently corresponded to the 50–90-kDa material obtained from PAPP-A/proMBP by reduction (Fig. 1A, lane 2). Sequence analysis confirmed that the PAPP-A subunit constituted the material in pool 1 and that proMBP, apart from a minor contamination with an approximately 500-residue fragment from PAPP-A, constituted the material in pool 2 (Table III).

By dot blot analysis carboxymethylated PAPP-A/proMBP reacted strongly with mAbs recognizing epitopes in the propeptide of MBP and in mature MBP (not shown). Using the same mAbs it was found in Western blot analysis (Fig. 1B, lane 3), that nonreduced PAPP-A/proMBP reacted poorly with both mAbs (lane 1), that the 215-kDa subunit of PAPP-A did not react at all, and that the 50–90-kDa material reacted strongly with both mAbs (lanes 2 and 4). MBP (Fig. 1B, lane 3) served as a control for mAb J6–8A4. Hence, consistent with the results described above, the 50–90-kDa material dissociated from PAPP-A/proMBP by reduction and carboxymethylation contains epitopes reacting with mAbs specific for MBP and its propeptide.

From the results presented above, it was likely that the

![Fig. 1. SDS-PAGE and Western blot analysis of PAPP-A and MBP. Panel A: lane 1, 8 µg of nonreduced PAPP-A/proMBP; lane 2, 10 µg of reduced PAPP-A/proMBP; lane 3, 0.5 µg of reduced MBP; lane 4, 3 µg of Sepharose CL-6B fraction 2 (Fig. 2). Panel B, Western blot analysis of the same samples using MBP mAb J6–8A4. Panel C, Western blot analysis of the same samples using MBP propiece mAb J6–8A4. Panel D, Western blot analysis of the same samples using MBP propiece mAb J6–8A4.](image)

**Table I**

| Tryptic peptide from reduced and carboxymethylated PAPP-A/proMBP | Sequence | Yield |
|---|---|---|
| TP1 (37–46) | DAFTLQVWLR | 18 |
| TP2 (130–154) | LLYNQAVATSVEQV(GGIFSPLTQK) | 13 |
| TP3 (386–413) | FNFDFGDGCDPENTQTC(FDPSPHR) | 11 |
| TP4 (427–459) | LDGSTHLNIFPAK | 27 |
| TP5 (496–528) | GISEIQSCSICMPEPFLTGCDLNIQDNTPAPK | 7 |
| TP6 (639–661) | ILVQYASNNASSP(CSPSGHWSMR) | 20 |
| TP7 (662–674) | EAEQHPDVEQGPK | 41 |
| TP8 (1179–1193) | SQTGSPTVTVTCEGK | 23 |
| TP9 (1289–1294) | VGSFCR | 46 |
| TP10 (1410–1443) | L4QPDOYAIQGSEATCSDLH(NEFILPMNVT) | 8 |
| TP11 (1444–1453) | DPHWHLNPT | 22 |
| TP12 (1457–1464) | VVCATAGLK | 40 |

**proMBP** (172–179) | FQWVDSR | 34 |
| **TproMBP** (210–214) | AHCLIR | 30 |
| **TproMBP** (215–222) | RLFFICSY | 16 |

\[a\] Details of peptide purification will be presented elsewhere (see footnote 2).

\[b\] The sequences of the residues in parentheses are taken from the cDNA sequence of PAPP-A (see footnote 2).

\[c\] Approximately 83 nmol of reduced and carboxymethylated PAPP-A/proMBP was digested.
commercial rabbit anti-PAPP-A-A230 would react with MBP. As shown in Fig. 1D, this was indeed the case when examined by a dot blot analysis. PAPP-A served as a positive control.

**DISCUSSION**

The finding presented here that proMBP is a constituent of circulating PAPP-A is the first demonstration of this molecule in serum. The conclusion that circulating PAPP-A is complexed with proMBP through disulfide bridge formation is based on the following concordant evidence. 1) Tryptic peptides originating from proMBP were found in a digest of reduced and carboxymethylated PAPP-A/proMBP; 2) a disulfide-bridged peptide connecting proMBP and PAPP-A and a peptide spanning the proMBP processing site have been isolated from nonreduced PAPP-A/proMBP in high yield; 3) sequence analysis shows that PAPP-A/proMBP contains three NH₂-terminal sequences, one originating from the PAPP-A subunit, and two originating from proMBP; 4) the PAPP-A subunit can be separated from the proMBP subunit by denaturing gel chromatography of reduced and carboxymethylated PAPP-A/proMBP; 5) mAbs directed against the propiece of MBP and against mature PAPP-A subunit by reducing SDS-PAGE or denaturing gel chromatography.

In the complex, one PAPP-A subunit is likely to be bound to one molecule of proMBP as judged from the yield of tryptic peptides, the yields of the NH₂-terminal residues upon sequence analysis of PAPP-A/proMBP, and the recovery of radioactivity (82 half-cystines in PAPP-A and 12 half-cystines in proMBP) in the two fractions obtained after denaturing gel chromatography.

Intriguingly, proMBP, when separated from the PAPP-A subunit after reduction, migrates in SDS-PAGE and elutes in denaturing gel chromatography as a species of 50-90 kDa, rather than as the expected 23-4 kDa polypeptide. The propiece being acidic (pl = 4.0) and MBP being basic (pl = 11) may enable proMBP to form very stable oligomers even after denaturation. In addition, since approximately 30 residues of glucosamine and 2-3 residues of galactosamine were found in the pool 2 material (not shown), the propiece is extensively glycosylated.

It has been suggested that the signal sequence of preproMBP comprises residues 1-15 or 1-16 (15, 16). However, the present results show that proMBP associated with PAPP-A contains NH₂ termini reflecting cleavage at Leu-17-His-18 and at Ala-16-Leu-17 (approximately 2:1 molar ratio). Both cleavage sites conform with the "-1 to -3 rule" (32), although Leu residues are rarely found in position -1 (33). Alternatively, the NH₂ terminus starting with His-18 may represent a secondary trimmed product.

Prior to or during storage in the granules of eosinophils phoglycosylated proMBP is cleaved at Gln-105-Thr-106 (15, 16). As discussed above, proMBP associated with PAPP-A is probably unprocessed. However, since MBP can be recovered from the placental septal fluid (23), processing can take place in certain compartments of the placenta.

The intracellular or extracellular compartments where the assembly of the PAPP-A/proMBP complex takes place are un-

---

**TABLE II**

| Peptide | Sequence |
|---------|----------|
| CBT1  | DCNYER (PAPP-A 380-385) + EETPCR |
| CBT2  | VVGIPQCQTCR (preproMBP 98-108) |

* Approximately 125 nmol of PAPP-A/proMBP was used for digestion. At present, half of the disulfide bridges in PAPP-A have been localized. The yields of peptides CBT1 and CBT2 are above average for peptides characterized so far.

Two sequences were present in equimolar yield. Only PTH-Glu was seen in cycle 2, and bis-PTH-Cys was seen in cycle 5, hence establishing the disulfide bridge.

c. The amino acid composition of both peptide sets agreed with the sequences determined.

Only one sequence was present. Bis-PTH-Cys was seen in cycles 7 and 10, hence establishing that the peptide is a dimer, in which Cys-104 in one mate is connected to Cys-104 in the other, and Cys-107 in one mate is connected to Cys-107 in the other.

---

**TABLE III**

| Sequence analysis of PAPP-A/proMBP, and of reduced and carboxymethylated Sepharose CL-6B pools |

The sequences were obtained from samples spotted on PVDF. Unequal amounts of the material in pools 1 and 2 were analyzed. The yields, corrected for the steady increase of background, are shown in parentheses (pmol).

| PAPP/A proMBP | Pool 1 | Pool 2 |
|---------------|--------|--------|
| Glu (63) | His (29) | Leu (26) | Glu (114) | His (21) | Leu (17) |
| Ala (78) | Leu (57) | His (12) | Ala (130) | Leu (35) | His (13) |
| Arg (79) | Arg | Leu (20) | Arg (96) | Arg (20) | Leu (16) |
| Gly (58) | Ser (20) | Arg (10) | Gly (90) | Ser (7) | Arg (11) |
| Ala (60) | Glu (32) | Ser (10) | Ala (88) | Glu (12) | Ser (4) |
| Thr (21) | Thr | Glu (23) | Thr (45) | Thr (4) | Glu (8) |
| Glu (40) | Ser (17) | Thr (9) | Glu (53) | Ser (5) | Thr (3) |
| Glu (50) | Thr (17) | Pro (50) | Glu (70) | Pro (50) | Phe (8) |

a. Sequence of the PAPP-A subunit.
b. Sequence starts at position 18 in proMBP.
c. Sequence starts at position 17 in proMBP.
d. The sequence Glu-Ala-Arg-Gly-Ala-Thr-Glu-Glu-Pro was also observed (less than 5 pmol level), reflecting the presence of a minor ~ 500-residue NH₂-terminal proteolytic fragment of PAPP-A (see Footnote 2).
known. Since MBP (14) and possibly also proMBP contain free cysteinyl SH-groups, the mechanism of complex formation probably involves thiol-disulfide exchange engaging one or more particularly exposed PAPP-A disulfide bridges. However, in view of the 1:1 stoichiometry discussed above, the assembly is likely to involve specific interactions between the two proteins.

It has been indicated previously that placental MBP is associated with large proteins (23, 24), and this investigation identifies PAPP-A as a binding protein for proMBP. The serum levels of MBP (approximately 0.28 μg/ml; Ref. 22) and PAPP-A (approximately 0.23 μg/ml subunit; Refs. 6 and 7) in late pregnancy determined by immunological methods suggest that essentially all MBP antigen present is bound to PAPP-A.

The commercial anti-PAPP-A A230 and other similar preparations have been used for determination of PAPP-A levels in pregnancy (2, 6, 7) and for immunohistochemical localization of PAPP-A in tissues (8–13). As shown here, it is polyspecific, also reacting with MBP and probably with proMBP. Hence, results based on the use of such preparations must be questioned.

The physiological significance of the interaction between PA-Pro-MBP and proMBP in pregnancy is not known, but the results reported here provide a novel basis for studying the biological roles of PAPP-A and placental MBP.

Acknowledgments—We thank the staff at the Department of Obstetrics and Gynecology, Aarhus University Hospital for donating pregnancy serum, P. A. Andreasen for gifts of mAbs, J. Checkel and D. A. Loegering for production of the mAbs to MBP and proMBP, and Lene Kristensen for excellent technical assistance.

REFERENCES
1. Lin, T.-M., Halbert, S.-P., Kiefer, D., Spellacy, W. N., and Gall, S. (1974) Am. J. Obstet. Gynecol. 118, 225–236
2. Bischof, P. (1979) Arch. Gynecol. 227, 315–326
3. Sutcliffe, R. G., Kakulas-Langlands, B. M., Coggins, J. R., Hunter, J. B., and Gore, C. H. (1986) Biochem. J. 211, 729–739
4. Davey, M. W., Teisner, B., Siniosich, M., and Grudzinskas, J. G. (1983) Anal. Biochem. 131, 18–24
5. Siniosich, M. J., Sim, R. B., and Teisner, B. (1990) Biochem. Int. 20, 579–580
6. Folkersen, J., Grudzinskas, J. G., Hindersens, P., Teisner, B., and Westergaard, J. (1981) Am. J. Obstet. Gynecol. 138, 910–924
7. Westergaard, J., Teisner, B., and Grudzinskas, J. G. (1983) Arch. Gynecol. 232, 211–216
8. Lin, T.-M., and Halbert, S. P. (1978) Science 195, 1249–1252
9. Tornhave, D., Channitza, J., Teisner, B., Folkersen, J., and Westergaard, J. (1984) Placenta 5, 427–432
10. Siniosich, M. J., Porter, R., Sloss, P., Bonifacio, M. D., and Saunders, D. M. (1984) J. Clin. Endocrinol. Metab. 64, 500–504
11. Sjöberg, J., Wahlström, T., Seppälä, M., Rutanen, E.-M., Kaitinen, R., Koskimies, A. I., Tenhunen, A., Sinosich, M. J., and Grudzinskas, J. G. (1984) Fertil. Steril. 41, 551–557
12. Bischof, P., Martin-Du-Pan, E., Lauber, K., Girard, J. P., Herrmann, W. L., and Sironenoke, P. C. (1983) J. Clin. Endocrinol. Metab. 56, 359–362
13. Sjöberg, J., Wahlström, T., Seppälä, M., Rutanen, E.-M., Kaitinen, R., Koskimies, A. I., Sinosich, M. J., Teisner, B., and Grudzinskas, J. G. (1984) Arch. Androl. 14, 253–261
14. Wasmoen, T. L., Bell, M. P., Loegering, D. A., Gleich, G. J., Prendergast, F. G., and McKeen, D. J. (1986) J. Biol. Chem. 261, 12559–12563
15. Barker, R. L., Gleich, G. J., and Pease, L. P. (1988) J. Exp. Med. 168, 1493–1498
16. McGrogan, M., Simonson, C., Scott, R., Griffith, J., Ellis, N., Kennedy, J., Campanelli, D., Nathan, C., and Gabay, J. (1988) J. Exp. Med. 168, 2255–2308
17. Gleich, G. J., Frigas, E., Loegering, D. A., Wasmøen, T. L., and Steinmuller, D. (1989) J. Immunol. 143, 2925–2927
18. Frigas, E., Loegering, D. A., Gleich, G. J. (1980) Lab. Invest. 42, 35–43
19. Hamann, K. J., Gleich, G. J., Gardel, R. H., and White, S. R. (1991) in The Airway Epithelium: Physiology, Pathophysiology, and Pharmacology (Farmer, S. G., and Hay, D. W. F. ed.) pp. 255–300, Marcel Dekker Inc., New York
20. Maddox, D. E., Butterfield, J. H., Ackerman, S. J., Coulam, C. B., and Gleich, G. J. (1983) J. Exp. Med. 158, 1211–1226
21. Maddox, D. E., Kophart, G. M., Coulam, C. B., Butterfield, J. H., Benirschke, K., and Gleich, G. J. (1984) J. Exp. Med. 160, 29–41
22. Wasmøen, T. L., Coulam, C. B., Leidman, K. M., and Gleich, G. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3029–3032
23. Wasmøen, T. L., McKeen, D. J., Benirschke, K., Coulam, C. B., and Gleich, G. J. (1989) J. Exp. Med. 170, 2051–2063
24. Wasmøen, T. L., Coulam, C. B., Benirschke, K., and Gleich, G. J. (1991) Am. J. Obstet. Gynecol. 165, 416–420
25. Gleich, G. J., Loegering, D. A., Mann, K. G., and Maldonado, J. E. (1976) J. Clin. Invest. 57, 633–640
26. Köhler, G., and Milstein, C. (1975) Nature 256, 495–497
27. Munch, M., Heegaard, C., Jensen, P. H., and Andreasen, P. A. (1991) FEBS Lett. 295, 102–106
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. Barholtz, V., and Jensen, A. L. (1989) Anal. Biochem. 177, 318–322
30. Sutrup-Jensen, I., Støphan, T. M., Jones, C. M., Leinhéd, P. B., Kristensen, T., and Wierich, M. D. (1984) J. Biol. Chem. 259, 8293–8303
31. Crimmins, D. L., Thoma, R. S., McCourt, D. W., and Schwartz, B. D. (1989) Anal. Biochem. 176, 255–260
32. von Heine, G. (1983) Eur. J. Biochem. 133, 17–21
33. von Heine, G. (1985) J. Mol. Biol. 184, 99–105