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BIOLOGICALLY ACTIVE DOMAIN IN SOMATOMEDIN-BINDING PROTEIN

Marja-Liisa Huhtala*[1], Riitta Koistinen, Pekka Palomäki*, Paul Partanen*, Hans Bohn***, and Markku Seppälä****

*Labsystems Research Laboratories, SF-00880 Helsinki, Finland

** Department I of Obstetrics and Gynecology, Helsinki University Central Hospital, SF-00290 Helsinki, Finland

*** Behringwerke AG, Marburg/Lahn, Federal Republic of Germany

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We have found that human decidua sythesizes a 34K somatomedin-binding protein PP12 (1-3). Purification of PP12 by immunochemical techniques from human placenta and adjacent membranes has also yielded lower-molecular weight immunoreactive polypeptides designated as PP12B (2). An individual 21K fragment of somatomedin-binding protein, and a mixture of fragments with molecular weight from 17K to 20K were isolated from this material using high performance liquid chromatography (HPLC). These fragments reacted with antibodies to native PP12 as shown by Western blotting. They all shared the same N-terminal amino acid sequence: Ala-Pro-Trp-Gln-, which is identical with that obtained for PP12. The 21K fragment was shown to bind somatomedin-C, or IGF-I (insulin-like growth factor-I). Since the N-terminal end of the 21K fragment is identical with that of the 34K somatomedin-binding protein, our results suggest that the 21K fragment is the N-terminal part of somatomedin-binding protein, and the somatomedin-binding domain resides in this N-terminal portion.

Placental protein 12 (PP12) was originally isolated from the human placenta and its membranes (2). Subsequent studies on tissue culture disclosed that the site of PP12 synthesis is secretory/decidualized endometrium and not placenta (3). In maternal serum, the highest levels of immunoreactive PP12 occur between 20 and 25 weeks of pregnancy (4). In amniotic fluid, the levels are 100-500 fold higher than in maternal serum (4). Analysis of the N-terminal sequence of PP12 has given a sequence of Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala (1), which is identical with somatomedin-binding protein isolated from human amniotic fluid (5). Purified placental PP12 also binds somatomedin-C. Both the somatomedin-binding protein of the human amniotic

[1] To whom correspondence should be addressed.

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fluid and PP12 are 34K proteins (1,5). Isolation of PP12 from human placenta has also yielded lower-molecular weight material (PP12B), which can be co-isolated with PP12 using polyclonal antibodies to PP12 (2).

In search for the somatomedin-binding domain of PP12 the present study was undertaken to characterize structural, antigenic and somatomedin-binding properties of the lower-molecular weight PP12-immunoreactive material.

MATERIAL AND METHODS

Isolation of PP12 and its fragments

Placental protein 12 and its fragments (PP12B) were isolated from human placenta and its adjacent membranes using immunochemical techniques as described previously (2). The fragments were further purified by reverse phase HPLC on a Vydak 218 TP 54 column (4.6 x 250 mm) using a linear gradient of acetonitrile (0-60 % in 30 min) in 0.1 % trifluoroacetic acid. The flow rate was 1 ml/min and detection was performed at 218 nm. The fraction size was 1 ml. The main protein fraction was rechromatographed under the same conditions.

Characterization of the HPLC-fractions

SDS-PAGE The HPLC-fractions were submitted to SDS-PAGE under reducing and non-reducing conditions using a 12% separation gel (6). Reduction was performed with 5 % 2-mercaptoethanol. Molecular weight markers were phosphorylase b (Mr=94,000), bovine serum albumin (Mr=67,000), ovalalbumin (Mr=43,000), carbonic anhydrase (Mr=30,000), soybean trypsin inhibitor (Mr=20,100) and alpha-lactalbumin (Mr=14,400). The gels were stained with Coomassie blue.

Immunoblotting The SDS-PAGE-separated proteins were transferred onto a nitrocellulose sheet and immunoreactivity of polypeptides was tested using rabbit anti-PP12 antiserum, followed by peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Copenhagen, Denmark). The enzyme reaction was developed using diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo) as described by Towbin et al. (7). Specificity of the anti-PP12 antiserum has been described earlier (2,4).

Edman degradation N-terminal sequences were determined by automated Edman degradation with gas-phase sequencer (model 470A Applied Biosystems Inc., Foster City, Ca, USA) (8,9). The HPLC-fractions (No. 22, 24 and 25) were applied directly to a Polybrene/sodium chloride-treated glass fiber filter and degraded in a sequencer using a CPTH program (Applied Biosystems standard sequencer programs). Phenylthiohydantoin derivatives were identified by reverse-phase HPLC on a Spherisorb column (S5 ODS 2) (4.6 x 250 mm) using a gradient of acetonitrile in 30 mM sodium acetate, pH 4.8 (10). Yields from the first cycle of Edman degradation were calculated from peak heights of HPLC chromatograms as compared with those of the standards.

Somatomedin-binding assay The binding experiments of somatomedin-C, or IGF-I (insulin-like growth factor-I), to the HPLC-purified fragments of PP12, were carried out as follows: wells of the microtitration plates (Microstrips^{R}, Eflab Oy, Helsinki, Finland) were first treated with a 0.2 % glutardialdehyde solution in 0.05 M sodium phosphate buffer (50 µl), pH 5.0 for 1 h at room temperature, and then washed twice with 0.05 M phosphate-buffered saline (0.9 % NaCl), pH 7.5 (PBS). The glutardialdehyde-treated wells were then coated with PP12B, or with its fragments, by incubating 0.1 ml PBS containing 2-4 µg/ml protein overnight at 4°C. The wells were washed once

with 0.1 % BSA in PBS. Then, 0.1 ml of 3 % BSA in PBS was added and incubated at 37°C for one hour. Washing was carried out three times with 0.5 % BSA in PBS. To study binding of IGF-I onto the protein-coated wells, 0.1 ml of (125 I) iodo-IGF-I (40,000 cpm) (Amersham Ltd, Bucks, UK) was added to the wells and incubated for 2 h at room temperature. The wells were then washed three times with the wash solution, cut out and counted in a gamma counter. Displacement of radio labeled IGF-I from PP12B, or from its fragments, was tested by the addition of 2 and 100 ng of unlabeled IGF-I (Amersham) to the incubation mixture together with labeled IGF-I.

RESULTS

Figures 1A and B show results of the HPLC-fractionation of PP12B. When rechromatographed the main protein fraction (No. 25) eluted as one peak at 47 % acetonitrile (Figure 1B). When this fraction was subjected to

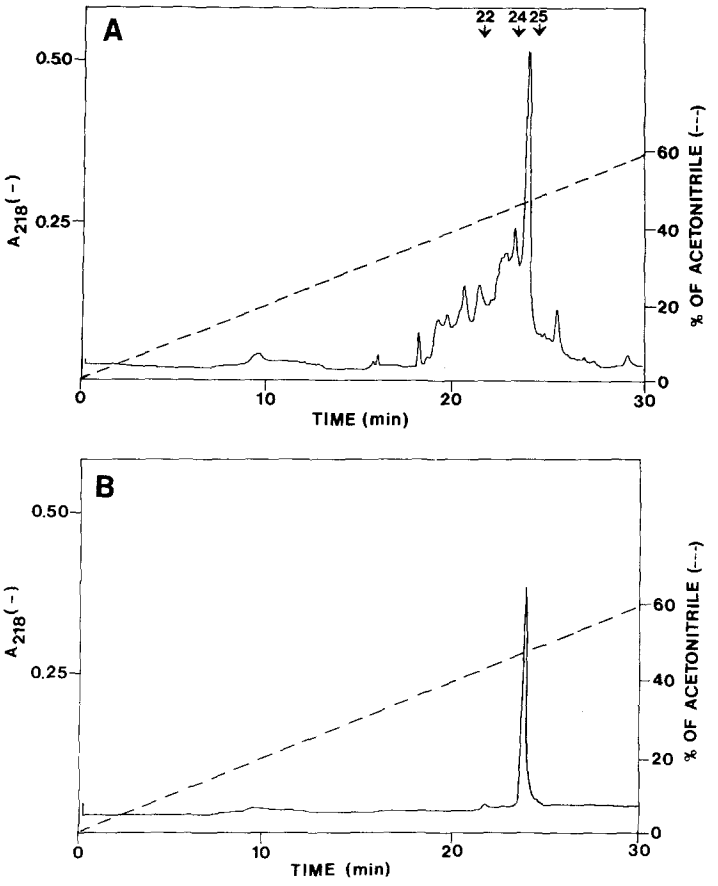

Figure 1A. High performance liquid chromatography of the crude preparation of PP12. The sample (50 µg) was injected into a reverse phase column. The column was eluted with a gradient of acetonitrile in 0.1 % TFA as indicated by broken line. The arrows indicate the point of fractions used for further characterization.

Figure 1B. Rechromatography of the main peak fraction (No. 25) from the previous step. Elution was carried out with the same gradient as in the first HPLC-run.

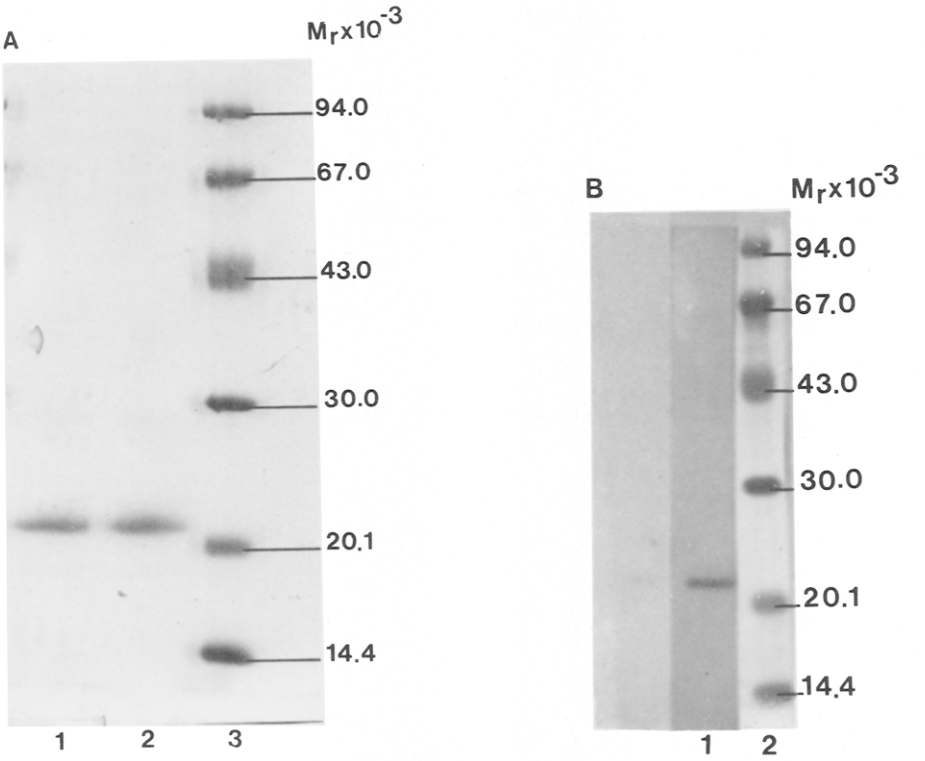

Figure 2A. SDS-PAGE of the HPLC-fraction No. 25. Lane 1, (3 µg) non-reduced. Lane 2, (3 µg) reduced with 5 % 2-mercaptoethanol. Lane 3, mol wt markers.

Figure 2B. Immunoblot analysis of fraction No. 25. Lane 1, HPLC-purified and reduced fraction No. 25 (0.2 µg) immunoblotted using anti-PP12 antiserum. Lane 2, mol wt markers.

analytical SDS-PAGE, it gave one band corresponding to molecular weight of about 21,000 (21K) both under reducing and non-reducing conditions (Figure 2A). A single PP12-immunoreactive band was seen in immunoblotting (Figure 2B). This band corresponded to the position of the protein band in SDS-PAGE. When two other protein fractions from HPLC (No. 22 and No. 24) were analyzed in SDS-PAGE, they gave apparent molecular weights from Mr=17,000 (17K) to Mr=20,000 (20K) (Figure 3A). The main components were consistently found at Mr=17K and =19K. Fraction No. 22 gave essentially one band corresponding to Mr=17K, with only trace amount of another band corresponding to Mr=19K. Reduction did not alter the electrophoretic mobility of any of these fragments and, therefore, reduced samples are shown only. These bands were also PP12-immunoreactive when immunoblotted (Figure 3B).

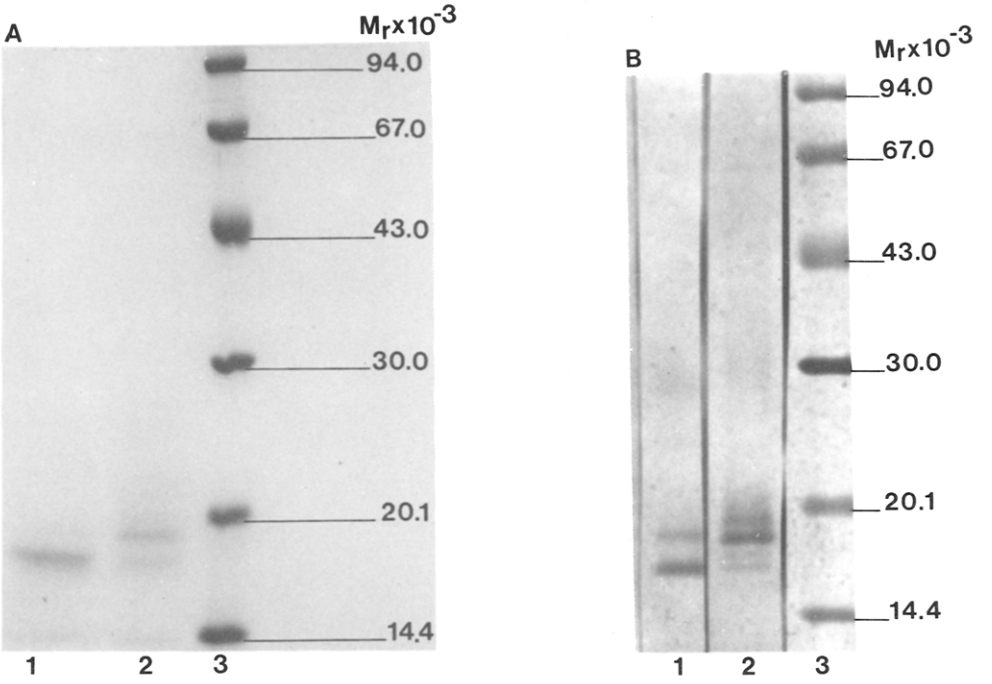

Figure 3A. SDS-PAGE of the reduced HPLC-fractions 22 and 24. Lane 1, fraction 22 (2 µg). Lane 2, fraction 24 (2 µg). Lane 3, mol wt markers.

Figure 3B. Immunoblot analysis of reduced HPLC-fractions No. 22 and No. 24. Lane 1, fraction No. 22 (0.3 µg). Lane 2, fraction No. 24 (0.3 µg). Lane 3, mol wt markers.

No polypeptides with the molecular weight of intact PP12 (Mr=34K) were detectable in these fractions.

A single N-terminal amino acid sequence of Ala-Pro-Trp-Gln- was obtained for the major HPLC-fraction (No. 25), as well as for the minor HPLC-fractions (No:s 22 and 24). Clear assignments were made for 13 of the first 15 residues of fraction No. 25. Positions 5 and 8 gave no ordinary phenylthio-

Table I

Results of Edman degradation of HPLC fractions

	1 5 10
Fraction 25	Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser-Ala
Fraction 24	Ala-Pro-Trp-Gln-
Fraction 22	Ala-Pro-Trp-Gln-Cys-Ala-Pro-Cys-Ser

The yield of alanine at cycle 1 was in excess of 35 % in each case.

Table II

Displacement of (125I)iodo-IGF-I binding to HPLC-fraction 25 by unlabeled IGF-I

Protein	IGF-I (ng/0.1 ml)	B %	B/Bo %
Fraction No. 25	0	9	100
	2	4.5	50
	100	0	0

Binding values are the mean of duplicate determinations.

hydantoin amino acids, but only indications of degradation products of the unstable S-phenylthiocarbamyl-phenylthiohydantoin derivative confirming cysteine at these positions. This N-terminal amino acid sequence is identical with that obtained for PP12 (1), or for the somatomedin-binding protein of human amniotic fluid (5) (Table 1).

Like PP12, the main protein fraction (No. 25) from HPLC with Mr=21K was found to bind IGF-I. Four hundred ng bound 9 % of labeled IGF-I added at the concentration of 100 pg/100µl. About 50 % displacement was achieved with 2ng of unlabeled IGF-I in each case (Table 2). This is roughly the same as reported for PP12 with Mr=34K (11). The other HPLC-fractions were not obtained in sufficient quantities to study binding activity.

DISCUSSION

The lower-molecular weight PP12-immunoreactive material from the human placenta and its membranes was found to be a convenient source of somatomedin-binding protein fragments. In this study we describe the isolation and characterization of these fragments.

The major protein peak from HPLC was considered homogeneous as evidenced by a single band with molecular weight of 21K in SDS-PAGE. This molecular weight is about 13K smaller than that obtained for PP12, or for the somatomedin-binding protein of amniotic fluid (1,5). Antibodies to PP12 reacted with the fragment as shown by immunoblotting indicating that it contains at least one identical antigenic determinant with PP12.

The crude preparation of PP12B also contained other PP12-immunoreactive fragments as shown by immunoblotting. The molecular weight of these fragments varied from 17K to 20K.

Primary structure analysis of these fragments suggests that they all are the N-terminal portions of the same protein, since their N-terminal amino acid sequences are identical. These fragments have apparently derived from the native PP12 as a result of proteolysis before and during purification from human placenta. Proteolysis of PP12 may also have taken place in vivo. Previous studies indicate that, in human serum, the molecular weight of somatomedin-binding proteins may vary from Mr=30K to Mr=150K (12,13). However, no molecular weight of less than 30K has been reported previously for any somatomedin-binding protein.

The somatomedin-binding capacity of the main HPLC-fraction was demonstrated after incubation of labeled IGF-I in microtitration plate wells coated with the fraction. Binding of radioactivity could be inhibited by the addition of unlabeled IGF-I to incubation mixture indicating specific binding of labeled IGF-I. It is not known whether the other N-terminal fragments are capable of binding IGF-I.

The immunological reactivity, functional properties, and the N-terminal sequences of the fragments leave little doubt that they are derived from the somatomedin-binding protein synthesized by decidua. These results also provide a background characterization of the IGF-I binding domain of somatomedin-binding protein.

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REFERENCES

1. Koistinen, R., Kalkkinen, N., Huhtala, M.-L., Seppälä, M., Bohn, H. & Rutanen, E.-M. (1986) Endocrinology 118, 1375.

2. Bohn, H. & Kraus, W. (1980) Arch. Gynecol. 229, 279.

3. Rutanen, E.-M., Koistinen, R., Wahlström, T., Bohn, H., Ranta, T. & Seppälä, M. (1985) Endocrinology 116, 1304.

4. Rutanen, E.-M., Bohn, H. & Seppälä, M. (1982) Am. J. Obstet. Gynecol. 144, 460.

5. Povoa, G., Enberg, G., Jörnvall, H. & Hall, K. (1984) Eur. J. Biochem. 144, 199.

6. Laemmli, U.K. (1970) Nature 227, 680.

7. Towbin, H., Staehhelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350.

8. Hewick, R.M., Hunkapiller, M.W., Hood, L.E. & Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990.

9. Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. & Hood, L.E. (1983) Methods Enzymolol. 91, 399.

10. Zimmerman, C.L., Appela, E., Pisano, J.J. (1977) Anal. Biochem. 77, 569.

11. Koistinen, R., Huhtala, M.-L., Stenman, U.-H. & Seppälä, M. (1986) submitted for publication.

12. Hintz, R.L. and Liu, F. (1977) J. Clin. Endocrinol. Metab. 45, 988-995.

13. Furlanetto, R.W. (1980) J. Clin. Endocrinol. Metabol. 51, 12.