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High homology between a trophoblastic protein (trophoblastin) isolated from ovine embryo and α-interferons

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Ovine trophoblastic protein B (oTPB), an embryonic protein, is a 20 kDa secretory protein which is synthesized by the ovine conceptus from days 12 to 22 of pregnancy. oTPB was purified by HPLC using ion-exchange chromatography on a DEAE column and was subsequently chromatographed on a reversed-phase column. Automated Edman degradation was then used to determine the N-terminal amino acid sequence up to 45 residues. The sequence data reveal a significant homology between oTPB and bovine interferons α of class II: 64% of the amino acids are identical and 75% are homologous. A highly conserved region including residues 23–44 exhibits 82% homology. Identity between oTPB and either HuIFN-α.9 or MuIFNα.1 is 55%. These alignments between oTPB and IFNs occur at the N-terminus of the mature proteins and proceed without deletion. These results suggest that oTPB is an embryonic interferon.

Amino acid sequence; Trophoblastin; Trophoblastic protein; Interferon; (Ovine embryo)

1. INTRODUCTION

In placental mammals, the establishment of pregnancy is mediated by multiple interactions between the embryo and its dam. Thus, maintenance of luteal progesterone secretion, which is essential to embryonic development, is controlled by the conceptus. In sheep, various physiological studies suggest that an embryonic proteinaceous component, named trophoblastin, has an antiluteolytic activity [1]. Subsequent data have indicated that this proteinaceous factor is secreted by the trophoblast during a short period extending from days 12 to 22 of pregnancy [1–3]. Among the numerous proteins synthesized in vitro by the ovine conceptus, one major secretory polypeptide is secreted during this same period [4,5]. This embryonic protein has been partially characterized and designated ovine trophoblastic protein 1 (oTP1) [4,16] or, alternatively, ovine trophoblastic protein B (oTPB) [5]. Both proteins show an apparent molecular mass of 20 kDa.

In order to gain further insights into the function of oTPB, we have purified this protein by high-performance liquid chromatography (HPLC) and determined its N-terminal amino acid sequence up to 45 residues. We have used computerized data banks to search for homologies between this sequence and those of other known proteins.

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2. MATERIALS AND METHODS

2.1. *Culture of embryos*

The studies were carried out in sheep of the breed Prealpes du Sud. The ewes were synchonized and superovulated as in [6]. 15-day-old embryos were recovered by flushing uterine horns with phosphate-buffered saline [7], transferred to sterile Petri dishes containing 15 ml Eagle's minimum essential medium (MEM) prepared as in [8] and cultured in an atmosphere of 5% CO_2 in air at 38°C on a rocking platform according to [4].

During the first 24 h of the incubation period, cultures were radiolabelled using 100 μCi of either L-[^{35}S]cysteine, L-[^{35}S]-methionine (Amersham, France), or L-[^{3}H]leucine (CEA, France) per culture dish. To enhance incorporation of the

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radioactive amino acid, the concentration of the respective unlabelled amino acid was limited to 1/15th the normal amount in the culture dish. At the end of this incubation, the embryos were discarded and the medium centrifuged at 10000 × g for 20 min at 4°C. The supernatant was frozen, lyophilized, and stored at −20°C.

2.2. *Purification of oTPB*

The lyophilized medium from one embryo was dissolved and dialysed against 0.05 M Tris-HCl buffer (pH 6.0) at 4°C in an ultrafiltration cell (Amicon 8MC) with a membrane (Filtron) of 10 kDa cut off. It was concentrated to 2 ml before being submitted to ion-exchange HPLC and reversed-phase HPLC on a Waters System.

Ion-exchange HPLC separations were carried out on a TSK DEAE 5PW anion-exchange column (75 × 7.5 mm i.d.) (Toyo Soda). The column was equilibrated with 0.05 M Tris-HCl buffer (pH 6.0) at a flow rate of 0.5 ml/min. After injection of the sample, a linear gradient was formed from 0 to 0.15 M KCl in 0.05 M Tris-HCl buffer (pH 6.0) within 40 min followed by isocratic elution at 0.15 M KCl in the same buffer for 20 min. Elution was monitored by the absorption at 280 nm. Fractions were collected every minute, measured for radioactivity by liquid scintillation counting, and submitted to electrophoresis.

oTPB fractions obtained by anion-exchange HPLC were directly applied to a TSK-TMS 250 protein reversed-phase column (75 × 4.6 mm i.d.) (Toyo Soda). Separation was carried out using a 40 min linear gradient from 80% solvent A (0.05% trifluoroacetic acid, TFA, in water) to 70% solvent B (0.05% TFA in acetonitrile) at a flow rate of 0.6 ml/min. Fractions containing oTPB were concentrated under vacuum in a Speed Vac concentrator and stored in 60% acetonitrile at −70°C until N-terminal sequencing.

2.3. *Characterization of oTPB*

Newly synthesized proteins, contained in radioactive fractions, were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (w/v) polyacrylamide gels [9]. Western blot analysis of purified oTPB was carried out according to [10]. The blot was probed with a specific rabbit anti-oTP1 serum (gift from Dr Michaël Roberts, University of Missouri and Dr Fuller Bazer, University of Gainsville, USA) followed by ^{125}I-labelled protein A. Radioactive proteins were localized by autoradiography and fluorography. M_r standards for the gels were purchased from Bio Rad.

2.4. *Determination of N-terminal amino acid sequence*

Ten 15-day-old embryos were necessary to obtain enough protein for the amino acid analyses. Automated Edman degradation of the purified oTPB was carried out using an Applied Biosystems 470A sequenator coupled with a model 120A phenylthiohydantoin (PTH) HPLC analyzer. Reagents and methods were those of the manufacturer according to [11]. The N-terminal sequence of the protein was performed on an approx. 1 nmol sample.

Cysteine residues were identified after either carboxymethylation with iodoacetic acid or by in vitro labelling of oTPB with L-[^{35}S]cysteine. The positions of leucine and methionine residues were also determined using oTPB labelled in vitro with either [^{3}H]leucine or [^{35}S]methionine. For this purpose, half of the PTH-amino acid samples were collected at each cycle and counted with an Aqualuma Plus scintillation cocktail using an Intertechnique SL 3000 counter.

2.5. *Sequence homology determination*

Homologies between the oTPB sequence and those of proteins in the NBRF library (release 13) or of putative open reading frames in sequences in Genbank (release 50) and EMBL (release 13) were investigated using Kanehisa's algorithm with the European database Bisance. Alignments were performed using the treatment of Kanehisa [12] with a window width of 10 and 2 uplets and selected with a maximal homology score of −60. All computations were carried out using the similarity matrix of Dayhoff [17].

3. RESULTS

3.1. *Purification of oTPB*

A typical elution profile of radiolabelled conceptus secretory proteins on the anionic exchange column is shown in fig.1. The two major radioactive peaks observed in fig.1 were analyzed by SDS-PAGE. The prominent radioactive peak which

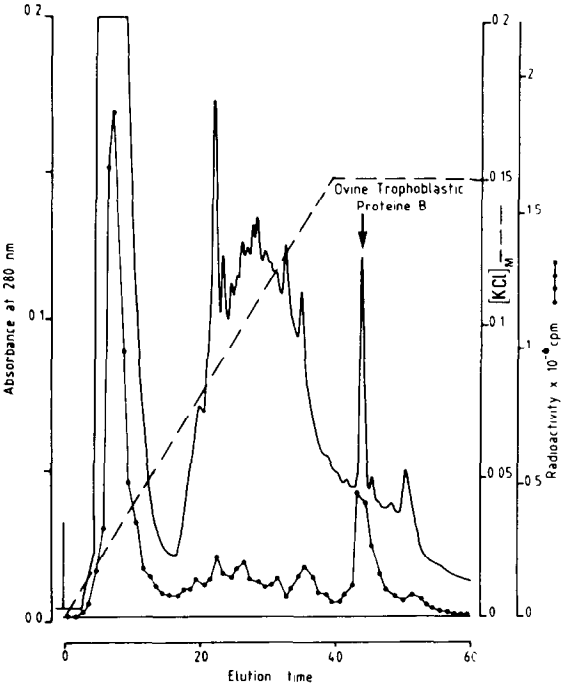


Fig. 1. Separation by ion-exchange HPLC of proteins from dialysed medium following 24 h incubation of ovine conceptuses. The column was DEAE-5PW (75 × 7.5 mm i.d.), and conditions were as given in section 2. Radioactivity in the collected fractions was measured by liquid scintillation counting using 10 μl of each fraction and 10 ml of Packard 2900 scintillator (---) KCl gradient. Elution time is given in min.

eluted in the first fractions of the HPLC gradient contained many bands of radioactivity but none which coincided with a protein of 20 kDa (fig.2A). In contrast, when the second peak, which coincided with a well-resolved protein peak eluting at 44 min, was analyzed a single radioactive band of apparent molecular mass 20 kDa was observed (fig.2B). This protein was identified as oTPB.

When oTPB fractions were analyzed by reversed-phase chromatography (fig.3) one prominent peak eluted from the column at an apparent acetonitrile concentration of 54%.

After immunoblotting of purified oTPB onto nitrocellulose and probing with an immuneserum against oTP1, a single immunoreactive band was observed (fig.2C), indicating that these two proteins are immunologically related.

3.2. *N-terminal amino acid sequence*

The amino acid sequence analysis was determined on oTPB after purification on reversed-phase chromatography. Two approaches were used. Firstly, purified oTPB was analyzed directly without any chemical modification. In this case a

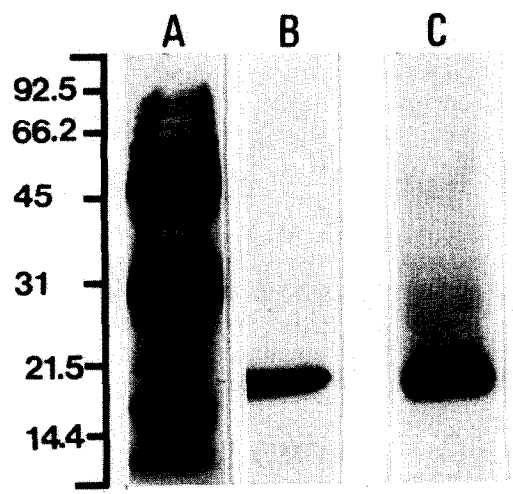


Fig. 2. Autoradiography of SDS-PAGE. Molecular mass (in kDa; shown on the left) markers used were phosphorylase *b* (92.5), bovine serum albumin (66.2), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5) and lysozyme (14.4). (A) Components of the first radioactive peak: fractions 3–5 from fig. 1; (B) ovine trophoblastic protein B: fractions 44, 45 from fig. 1; (C) Western blot of oTPB purified by reversed-phase HPLC and probed with immune serum against oTP1. The immunocomplex was revealed with ^{125}I-protein A.

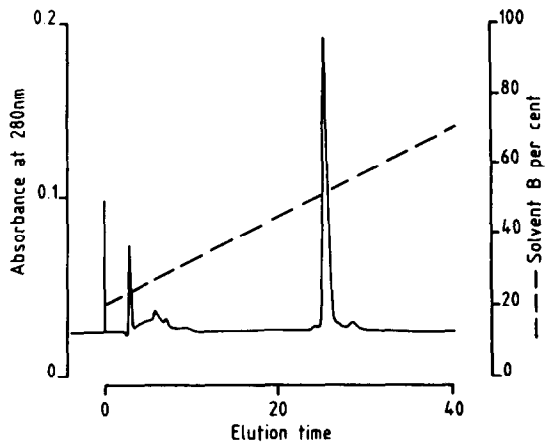


Fig. 3. Reversed-phase HPLC chromatography of pooled fractions 44, 45 from fig. 1. oTPB was eluting using a 40 min linear gradient from 80% solvent A (0.05% TFA in water) to 70% solvent B (0.05% TFA in acetonitrile).

single amino acid sequence of 45 residues was determined (fig.4). Secondly, oTPB labelled in vitro with either L-[^{3}H]leucine, L-[^{35}S]methionine, or L-[^{35}S]cysteine was sequenced in order to confirm the position of these residues in the first 25 amino acids (fig.5). Moreover, the cysteine residue at position 1 was also confirmed by sequencing with carboxymethylated oTPB. Assignment of cysteine at position 29 in oTPB was made on the basis of the lack of a detectable PTH derivative using untreated protein.

Computer comparison of the 45-amino-acid long N-terminal sequence of oTPB reveals a high homology with the N-terminal sequence of α-interferons of different species (bovine, murine, human) (fig.4). The best percent match (63.6%) is with bovine α-interferon class II (BoIFN-αII.1) [13] giving a homology score of -160. A higher homology is found (73%) when conservative amino acid substitutions are taken into account following the Dayhoff classification. Moreover, a long run of highly conserved residues is observed from positions 23 to 44 corresponding to a strict homology of 82%. A slightly lower homology is found with human α_9-interferon (HuIFN-α.9) [14] and mouse α_1-interferon (MuIFN-α.1) [15], both of which exhibit a percent match of 55.6%, with 25 out of 45 residues being identical. However MuIFN-α.1 gives a better homology score than HuIFN-α.9 (-140 instead of -137).

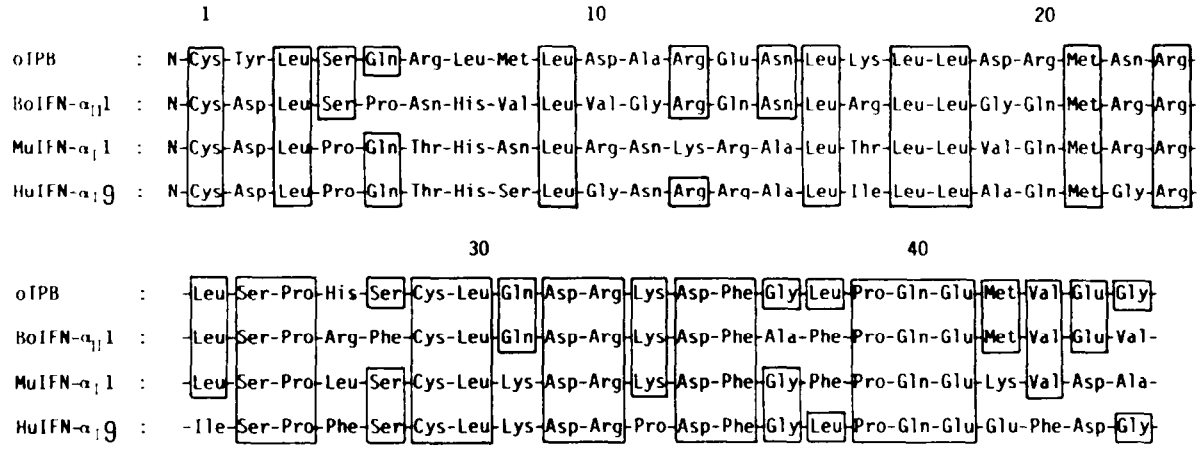
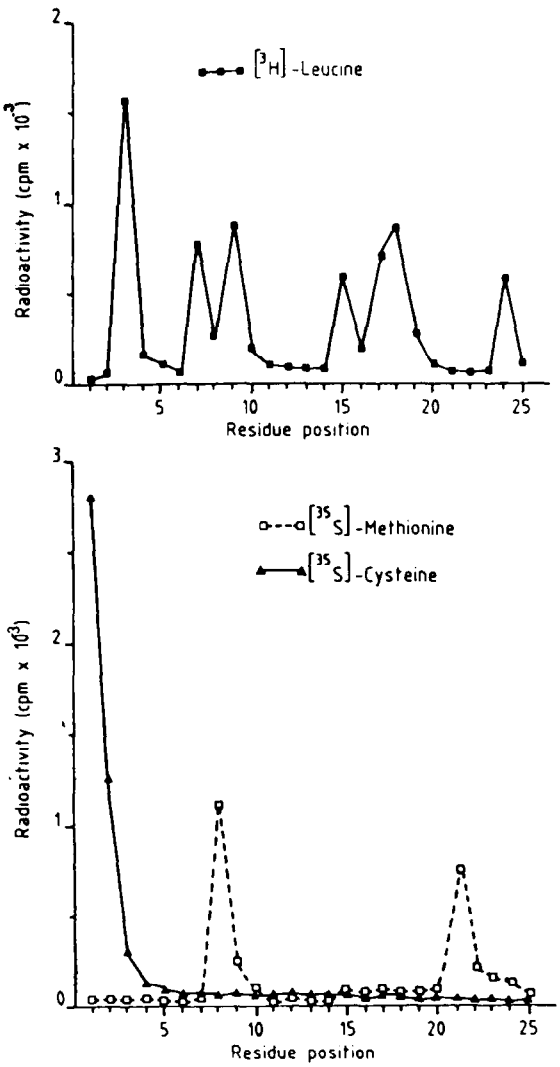


Fig. 4. Comparison of the 45 amino acid N-terminal sequence of ovine trophoblastic protein B (oTPB) with those of bovine interferon II.1 (BoIFN-II.1), murine interferon 1 (MuIFN-α1) and human interferon 1(HuIFN-α9). Closed boxes indicate identical residues between oTPB and interferons.



4. DISCUSSION

Purification of oTPB by HPLC was achieved in a single ion-exchange purification step on a DEAE column. Electrophoretic analysis of the column fractions and subsequent reversed-phase chromatography by HPLC confirmed that the purification method was sufficient to give oTPB in pure form. The degree of purity obtained for oTPB permitted the unambiguous determination of the N-terminal sequence up to 45 amino acids.

Immunoblotting analyses established the immunological relationship between oTPB and oTPI. These two proteins exhibit the same apparent molecular mass [4,5] and the same pattern in isoelectrofocusing ([4] and unpublished). The fact that oTPB was late eluting in the salt gradient on the anion-exchange resin confirms its acidic nature. All these data suggest that oTPB and oTP1 are identical. The two proteins are secreted in major amounts during the same short period [4,5,7] like the antiluteolytic activity of the proteinaceous component named trophoblastin [1]. It has also been shown that oTP1 presents antiluteolytic activity [16]. Hence, it may be assumed that these three factors, oTPB, oTP1 and trophoblastin, are the same embryonic component.

Fig. 5. N-terminal 25 amino acid sequence of oTPB labelled in vitro with either L-[^{35}S]cysteine, L-[^{35}S]methionine, or L-[^{3}H]leucine. Positions of radioactive residues confirm the amino acid sequence obtained with unlabelled oTPB (fig. 4).

Homologies observed between oTPB and α-interferons reach 73%. Moreover, the alignment of the amino acid sequences occurs at the N-terminus of mature IFNs and proceeds without deletion. These data reinforce the significance of similarities between oTPB and α-interferons. It is apparent from comparison of the N-terminal sequence of oTPB with different members of the α family of interferons that oTPB is significantly more homologous to BoIFN-αII.1 than to HuIFN-α.9 or MuIFN-α.1. HuIFN-α.9 and MuIFN-α.1 correspond to the first subfamily (class I) genes and BoIFN-α.1 to the second distinct subfamily (class II) [13]. It may be suggested that oTPB is more closely related to class II than to class I of α-interferons.

All these results of structural analysis strongly suggest that oTPB is an embryonic interferon-like molecule. Work is in progress to determine whether oTPB has any of the biological activities of interferon. Provided that analogies between oTPB and IFNs are confirmed by further studies, oTPB could be the first isolated α-interferon of class II naturally expressed in a mammalian tissue.

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REFERENCES

[1] Martal, J., Lacroix, M.C., Loudes, C., Saunier, M. and Wintenberger-Torrès, S. (1979) J. Reprod. Fert. 56, 63–73.
[2] Heyman, Y., Camous, S., Fèvre, J., Méziou, W. and Martal, J. (1984) J. Reprod. Fert. 70, 533–540.
[3] Martal, J., Charlier, M., Charpigny, G., Camous, S., Chêne, N., Reinaud, P., Sade, S. and Guillomot, M. (1987) Livest. Prod. Sci. 17, 193–210.
[4] Godkin, J.D., Bazer, F.W., Moffatt, J., Sessions, F. and Roberts, R.M. (1982) J. Reprod. Fert. 65, 141–150.
[5] Martal, J., Charlier, M., Camous, S., Fèvre, J. and Heyman, Y. (1984). in: 10th International Congress on Animal Reproduction and Artificial Insemination, Urbana University of Illinois, vol. III, abstr. 509.
[6] Fléchon, J.E., Guillomot, M., Charlier, M., Fléchon, B. and Martal, J. (1986) Reprod. Nutr. Dev. 26, 1017–1024.
[7] Tervit, H.R., Whittingham, D.G. and Rowson, L.E.A. (1972) J. Reprod. Fert. 30, 493–497.
[8] Basha, S.M.M., Bazer, F.W. and Roberts, R.M. (1980) J. Reprod. Fert. 60, 41–48.
[9] Laemmli, U.K. (1970) Nature 277, 680–685.
[10] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[11] Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990–7997.
[12] Kanehisa, M. (1984) Nucleic Acids Res. 12, 203–213.
[13] Capon, D.J., Shepard, H.M. and Goeddle, D.V. (1985) Mol. Cell. Biol. 5, 768–779.
[14] Lawn, R.M., Adelman, J., Dull, T.J., Grass, M., Goeddle, D. and Ullrich, A. (1981) Science 212, 1159–1162.
[15] Shaw, G.D., Boll, W., Taira, H.G., Mantei, N., Lengyel, P. and Weissman, C. (1983) Nucleic Acids Res. 11, 555–573.
[16] Godkin, J.D., Bazer, F.W., Thatcher, W.W. and Roberts, R.M. (1984) J. Reprod. Fert. 71, 57–64.
[17] Dayhoff, M.O., Schwartz, R.M., Orcutt, B.C. (1978) in: Atlas of Protein Sequence and Structure, vol. 5, suppl. 3, pp. 345–352, National Biomedical Research Foundation, Washington, DC.