Rapid Communication

Differential glycosylation of the components of the bovine trophoblast protein-1 complex

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Summary

The bovine trophoblast protein-1 complex, a major secretory product of the day 17 to 18 conceptus, has been implicated in extension of luteal lifespan during early pregnancy. This glycoprotein complex, identifiable by immunoprecipitation procedures utilizing rabbit antiserum to ovine trophoblast protein-1, exists as seven isomers of two size classes (22 and 24 kDa). Culture of embryos with tunicamycin demonstrated that the isomers are N-linked glycoproteins, as deglycosylated products migrate as a single band (18 kDa) during electrophoresis. Culture with deoxymannojirimycin indicated that the 24 kDa form is complex in nature, whereas treatment with endoglycosidase H and lectin chromatography indicated that the 22 kDa form is a high-mannose type glycoprotein. These results indicate that molecular weight variants of bovine trophoblast protein-1 arise as a single translation product that undergoes differential post-translational glycosylation.

Introduction

The presence of a functional corpus luteum is essential for pregnancy maintenance in the cow and ewe. The conceptus, therefore, must signal its presence to attenuate uterine prostaglandin F₂α secretion that would otherwise lead to the demise of the corpus luteum, termination of pregnancy, and resumption of ovarian cyclicity. For both the cow and ewe, signaling by the conceptus occurs via secretion of proteins (Godkin et al., 1984; Knickerbocker et al., 1986). The 'signal' proteins are similar for both species since interspecies transfer of trophoblastic vesicles between the ewe and cow will cause cycle extension (Martal et al., 1984). In sheep, the anti-luteolytic molecule has been identified as ovine trophoblast protein-1 (oTP-1; Godkin et al., 1984). Cattle secrete immunologically cross-reactive molecules similar to oTP-1 called the bovine trophoblast protein-1 (bTP-1) complex. This complex consists of seven isomers secreted in two size classes (Helmer et al., 1987). These molecules appear to be members of the α-interferon family (Imakawa et al., 1987; Stewart et al., 1987) and likely represent a novel role for interferon molecules.

While oTP-1 is not glycosylated (Anthony et al., 1988), bTP-1 is glycosylated since embryos cultured with [³H]glucosamine incorporated the radioisotope into bTP-1 (Anthony et al., 1988). In addition, the primary translation product of bovine
mRNA, which migrated as a single 17–18 kDa protein species during gel electrophoresis, was smaller than its secreted form (22 and 24 kDa; Helmer et al., 1987; Anthony et al., 1988). Therefore, the maternal recognition of pregnancy signal from the cow, bTP-1, has undergone evolutionary divergence from the nonglycosylated ovine signal and other related interferons which are nonglycosylated, O-glycosylated and rarely N-glycosylated (Langer and Pestka, 1985; Bielefeldt Ohmann et al., 1987). In the following series of experiments, we demonstrate that bTP-1 is glycosylated in an N-linked manner and that differences in molecular weight between classes of bTP-1 are due to differential post-translational processing to form high-mannose and complex carbohydrate species of bTP-1.

Materials and methods

Materials. L-[35S]Methionine was from Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA); endo-α-N-acetylglucosaminidase H (Endo H) from Streptomyces plicatus was purchased from Miles Laboratories (Naperville, IL); deoxymannojirimycin-HCl (DMM) from Bacillus species was obtained from Boehringer Mannheim (Indianapolis, IN); concanavalin A-Sepharose 4B and tunicamycin were from Sigma (St. Louis, MO); neuraminidase from Vibrio cholerae was from Life Technologies and endo-β-N-acetylgalactosaminidase (O-glycanase) from Diplococcus pneumoniae was purchased from Genzyme (Boston, MA). All other materials were supplied as noted previously (Helmer et al., 1987) or were reagent grade or better.

In vitro culture of conceptuses. Conceptuses were collected from Angus and Brangus cows at day 17–18 of pregnancy and were cultured as previously described (Helmer et al., 1987) for 72 h with fresh Eagle’s minimum essential medium (MEM) being replaced every 24 h. Cultures were carried out with methionine-deficient (0.1 × ) medium supplemented with 50–100 μCi [35S]methionine/culture/24 h. Conceptus-conditioned medium was centrifuged for 15 min at 2600 × g to remove particulate matter and dialyzed (Mr cutoff = 3500) extensively to remove low-molecular-weight compounds. Immunoprecipitation (IMP) of proteins in medium was carried out as previously described (Helmer et al., 1987). One-dimensional and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1-D and 2-D SDS-PAGE), using 12.5% (w/v) polyacrylamide gels, were performed as described elsewhere (Roberts et al., 1984). Radioactive polypeptides were detected by fluorography using Kodak XAR film.

Results

Inhibition of glycosylation during in vitro culture of conceptuses. A bovine conceptus was incubated with L-[35S]methionine in the presence of tunicamycin (20 μg/ml) to inhibit N-linked glycosylation. Without tunicamycin, two molecular weight classes of bTP-1 could be immunoprecipitated from conditioned culture medium (22 and 24 kDa). In the presence of tunicamycin, the con-
ceptus secreted only one species ($M_f = 18$ kDa) that could be immunoprecipitated from culture medium (Fig. 1). A conceptus was also cultured in the presence of DMM (1 mM) for 24 h to inhibit complex glycoprotein formation. Only one molecular weight species of bTP-1 (22 kDa) was detected in culture supernatants from the DMM-treated conceptus (Fig. 2). The 24 kDa form of bTP-1 was absent.

**Glycosidase treatment of conceptus proteins.** Conceptus culture supernatants were incubated for 48 h with Endo H, which cleaves the two N-acetylglucosamine residues of the chitobiose core of high mannose, N-linked glycoproteins (Tarentino et al., 1974). Treatment with Endo H resulted in a shift in molecular weight of the 22 kDa form of bTP-1 to approximately 20 kDa (Fig. 3) but had no effect on the electrophoretic mobility of the 24 kDa form of bTP-1. In a parallel experiment, conceptus culture supernatant from the first 24 h of culture was incubated in the presence of neuraminidase (0.1 U/50 µl) with or without O-glycanase (2.8 mU/50 µl) to assess whether bTP-1 contained sialic acid residues or any O-linked carbohydrate chains (Lamblin et al., 1984). Neither of the enzyme treatments altered the molecular weights or isoelectric points of proteins in the bTP-1 complex as discerned by electrophoretic migration following 1-D or 2-D SDS-PAGE (data not shown).

**Concanavalin A-Sepharose column chromatography.** Conceptus-conditioned culture medium was passed over a 2 ml concanavalin A-Sepharose 4B column. Although the bulk of the 24 kDa form of

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**Fig. 2.** Fluorograph of SDS-PAGE of conceptus supernatants from embryos cultured in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of DMM (1 mM) for 24 h. Conceptuses preincubated for 24 h were subsequently cultured with DMM for 24 h in methionine-deficient medium. After the first 4 h of culture, 100 µCi L-[^35S]methionine was added to culture medium. After 24 h, tissue and medium were separated and medium was dialyzed extensively against distilled water and lyophilized. Aliquots of medium were immunoprecipitated with either antiserum to oTP-1 (lanes 3 and 5) or normal rabbit serum (lanes 4 and 6) as previously described (Helmer et al., 1987) and separated by SDS-PAGE in the presence of 2-mercaptoethanol.

**Fig. 3.** Fluorograph of SDS-PAGE of conceptus proteins treated with Endo H. The experiment utilized conceptus supernatants from the second 24 h of culture in which conceptuses had been cultured in methionine-deficient MEM supplemented with 100 µCi L-[^35S]methionine. After medium was dialyzed and lyophilized, samples were resolubilized with 0.47 ml of 0.1 M NaPO₄, pH 6.1, 30 mM EDTA, 0.3% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 mM PMSF and 0.02% (w/v) NaN₃. Aliquots were incubated at 37°C in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of Endo H (6 µg, 30 µl) for 24 h. At this time, Endo H (6 µg, 30 µl) was added again to the appropriate samples and incubations were continued for a further 24 h at 37°C. Enzyme digestions were stopped by boiling samples for 3 min. Samples were then immunoprecipitated using either antiserum to oTP-1 (lanes 3 and 5) or normal rabbit serum (lanes 4 and 6) and resolved on 12% polyacrylamide gels in the presence of 2-mercaptoethanol. Fluorographs were exposed to the dried gel for various times to maximize detection of individual proteins present.
bTP-1 passed freely through the column, none of the 22 kDa form did so (Fig. 4). No appreciable amount of radioactive material was removed from the column by elution with 0.1 M α-methyl-D-glucoside or 0.1 M α-D-methyl-mannoside (data not shown). Elution with 6 M guanidine-HCl, pH 3.1, resulted in elution of all of the 22 kDa form of bTP-1. A small proportion of the 24 kDa form of bTP-1 was released from the column; this represented a very small fraction of the 24 kDa form found in crude culture medium or when compared to material not binding to the column (Fig. 4).

**Discussion**

It has been shown that a component of conceptus secretory proteins is responsible for extension of luteal lifespan in the cow and ewe (Godkin et al., 1984; Knickerbocker et al., 1986). Extension of luteal lifespan in the ewe is due to secretion of oTP-1 by the conceptus (Godkin et al., 1984), a molecule which is immunologically similar to the bovine conceptus product called bTP-1 (Helmer et al., 1987). These immunologically related proteins differ in size (oTP-1, \( M_r = 18 \) kDa; bTP-1, \( M_r = 22 \) and 24 kDa) and isoelectric variants (oTP-1, \( pI = 5.4-5.7 \); bTP-1, \( pI = 6.5-6.7 \) (Helmer et al., 1987)). Whereas oTP-1 is not glycosylated (Anthony et al., 1988), the bovine molecule is, in a previously undescribed manner.

Culture of bovine conceptuses with tunicamycin, an inhibitor of N-linked glycosylation (Tkacz and Lampen, 1975), resulted in loss of immunoprecipitable bTP-1 in the 22–24 kDa range with concomitant appearance of immunoreactive molecules migrating at 18 kDa. These results indicate that both forms of bTP-1 are N-linked. These data are also consistent with molecular weight estimates of bTP-1 produced from translation of bovine conceptus mRNA by wheat germ lysate (\( M_r = 17-18 \) kDa (Helmer et al., 1987; Anthony et al., 1988)). Conceptus secretory proteins were also incubated with O-glycanase, an enzyme which cleaves carbohydrates that are linked to the polypeptide chain via serine or threonine (Lamblin et al., 1984). Conceptus secretory proteins were first incubated with neuraminidase, since removal of terminal sialic acid residues is required for O-glycanase to be effective. Neuraminidase and O-glycanase were effective in removal of sialic acid residues and cleavage of O-linked chains, respectively, from control glycoproteins (fetuin, asialofetuin; data not shown), but enzyme treatments had no effect on bTP-1. Therefore, bTP-1 does not contain O-linked carbohydrate moieties or detectable quantities of terminal sialic acid residues.

Several lines of evidence indicate that the two molecular weight classes of bTP-1 arise from post-translational processing of a common transcript (18 kDa) to form a 22 kDa high-mannose form and a 24 kDa complex-type form. First, culture of conceptuses in the presence of DMM, an inhibitor of mannosidase I (Fuhrmann et al., 1984), which causes processing of high-mannose forms to complex-type oligosaccharides, blocked synthesis of the 24 kDa form, but not the 22 kDa form of bTP-1. Second, treatment of conceptus secretory proteins with Endo H resulted in reduction of the 22 kDa form of bTP-1 to 20 kDa but did not affect the 24 kDa form. Finally, experiments utilizing a concanavalin A-Sepharose column to bind high-mannose units (Cummings and Kornfeld, 1982), indicated that the majority of the 24 kDa form of bTP-1 did not bind to the column.
Conversely, all of the 22 kDa form of bTP-1 was bound to concanavalin A-Sepharose and could only be removed by elution with 6 M guanidine-HCl. A small proportion of the 24 kDa form did bind to the column, perhaps because of some nonspecific binding or because a subpopulation of the 24 kDa form may contain sufficient numbers of mannose moieties to allow some binding to concanavalin A-Sepharose.

Glycosylation of bTP-1 is unusual for several reasons. The related molecule, oTP-1, is not glycosylated. Secondly, oTP-1 and probably bTP-1 are members of the α-interferon family (Imakawa et al., 1987; Stewart et al., 1987), and few α-interferons are glycosylated. Pestka reported that all but one species of human α-interferon lack the Asn–X–Ser/Thr sequence necessary for N-glycosylation and that O-glycosylation is more common for α-interferons (Pestka et al., 1987). Only one site exists on oTP-1 for N-glycosylation (Imakawa et al., 1987). The glycosylation of bTP-1 may reflect simple evolutionary divergence. Alternatively, its glycosylation may be important for increasing the half-life and stability of bTP-1 once secreted from the conceptus into the uterine lumen. It could also affect binding of bTP-1 to its endometrial receptor or initiation of its biological effects. Finally, glycosylation of bTP-1 is of interest because the differential processing documented here appears to account for the molecular weight variants in the bTP-1 complex of proteins. It remains to be determined whether isoelectric and molecular weight variants have differing biological properties.

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References