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N-GLYCOSYLATED AND UNGLYCOSYLATED FORMS OF CAPRINE TROPHOBLAST PROTEIN-1 ARE SECRETED BY PREIMPLANTATION GOAT CONCEPTUSES

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Summary. Goat conceptuses secrete caprine trophoblast protein-1 (cTP-1) which is related antigenically to the abundant embryonic interferon-α_{II} of sheep and cattle. Antiserum to ovine and bovine TP-1s immunoprecipitated three molecular weight classes (23,000, 21,000 and 17,000, each with two isotypes) of cTP-1 from goat conceptus culture medium. Cultures which contained tunicamycin resulted in a shift in the M_r=23,000 and M_r=21,000 forms to a M_r of 17,000. The M_r=23,000 and 21,000 forms, but not the M_r=17,000 form, bound to Concanavalin A-Sepharose and were eluted under conditions selective for glycoproteins bearing complex-type oligosaccharide(s). Thus cTP-1 is a mixture of glycosylated and unglycosylated polypeptides.

Ovine and bovine blastocysts secrete trophoblast protein-1 (oTP-1 and bTP-1, respectively), an abundant low molecular weight acidic polypeptide complex, to signal the impending pregnancy to the mother (1,2). TP-1 belongs to the interferon-α gene family (3-6) and has interferon-like anti-viral and anti-proliferative activities (7,8). Pregnancy recognition is mediated through binding of TP-1 to uterine interferon receptors (9) resulting in an alteration of endometrial protein and prostaglandin metabolism (10,11).

Human interferon-α is a gene family of multiple gene products with pleiotropic effects (12). The oTP-1 complex of four isoelectric variants (M_r=17,000), like most interferon-α's (12), is not glycosylated (13). bTP-1 is heterogeneous with respect to charge and mass (M_r=24,000 and 22,000) (14,15). Both molecular weight classes of bTP-1 are glycosylated (15).

Antiserum to oTP-1 reacts with a secretory product of the preimplantation goat conceptus, tentatively named cTP-1 (16). cTP-1 is

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Abbreviations used: oTP-1, bTP-1, and cTP-1: trophoblast protein-1 from ovine, bovine, and caprine conceptuses, respectively; ConA: Concanvalin A; 1D and 2D SDS-PAGE: one-and two-dimensional polyacrylamide gel electrophoresis, respectively, in the presence of sodium dodecyl sulfate.

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expressed during the period of maternal recognition of pregnancy in the goat, days 17-21 (16). An oTP-1 cDNA hybridized to RNA from day 17 goat conceptuses (6). cTP-1 had three molecular weight variants (M_r of 23,000, 21,000, and 17,000) each with two isotypes, for a total of six polypeptides (16). Here, we demonstrated that cTP-1 was a mixture of nonglycosylated and glycosylated polypeptides bearing N-asparaginyl oligosaccharides.

Methods

Twelve crossbred female goats were bred on the first day of estrus (day 0) and day 17 conceptuses were collected as described previously (16). Conceptuses were incubated in 15 ml of Modified Eagle's Medium containing 5 μCi/ml of L-[^{35}S]-methionine (1200 Ci/mmol; New England Nuclear, Boston, MA). To the medium of eight cultures was added tunicamycin (Sigma Chemical Co, St. Louis, MO) from a 10 mg/ml stock solution in dimethylsulfoxide at final concentrations of 5 (n=4 conceptuses) and 10 μg/ml (n=4). To medium of four cultures was added dimethylsulfoxide only. Incubation was performed at 37^{o}C for 20-24h in an atmosphere of 5% CO_2 in 50% O_2 and 45% N_2 on a rocking platform (16). Medium was separated from tissue by centrifugation (12,000 x g, 20 min). Tissue was extracted into 5 ml of 50 mM Tris-acetate, pH 7.5, 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 2% (v/v) Nonidet P40, and 0.02% (w/v) sodium azide. Soluble protein in tissue extracts was harvested from the supernatant after centrifugation (30,000 x g, 30 min). Medium or soluble tissue protein was incubated for 20 h at 4^{o}C with 50 μl of rabbit antiserum to oTP-1 (1) or bTP-1 (14). Immune complexes were collected onto 200 μl of 10% (v/v) Protein A-Sepharose.

Medium (5.0 ml) from a culture unsupplemented with tunicamycin was applied to a 0.5 x 2 cm column of ConA-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in 50 mM Tris-acetate, 0.14 M NaCl, 0.1% (v/v) Nonidet P40, 0.1 mM $MnCl_2$ and 0.1 mM $CaCl_2$ (17). Stepwise elution employed 10 mM α-methylglucoside (at 22^{o}C) followed by 100 mM α-methylmannoside (at 60^{o}C) in the same buffer (17). cTP-1 was immunoprecipitated from each fraction by using antiserum to bTP-1 as described above.

Protein in medium and immunoprecipitates were analyzed by 1D (18) or 2D (19) SDS-PAGE in 12.5% polyacrylamide. Fluorograms were prepared as described previously (19). Fluorograms were scanned using an LKB UltroScan Densitometer and data were analyzed using the GelScan-XL software version 3.01 (LKB, Bromma, Sweden). Aliquots of medium were assayed for trichloroacetic acid precipitable radioactivity. Results were analyzed by the General Linear Model of the Statistical Analysis System (20). The Student-Newman-Keuhls test was used to test for significance.

Results

Proteins secreted by goat conceptuses during in vitro culture included a low molecular weight, acidic group of polypeptides as displayed by 2D-PAGE and fluorography (Fig. 1A). The cTP-1 complex (16), was comprised of at least 6 polypeptides with three molecular weights of 23,000, 21,000 and 17,000. At each of these molecular weights were a pair of isotypes. Supplementation of conceptus cultures with tunicamycin (10 μg/ml) resulted in an apparent increase in the electrophoretic mobility of the two higher molecular weight groups to an apparent mass of 17,000 (Fig. 1B). In all, four isotypes were apparent. Tunicamycin did inhibit incorporation of [^{35}S]-methionine into

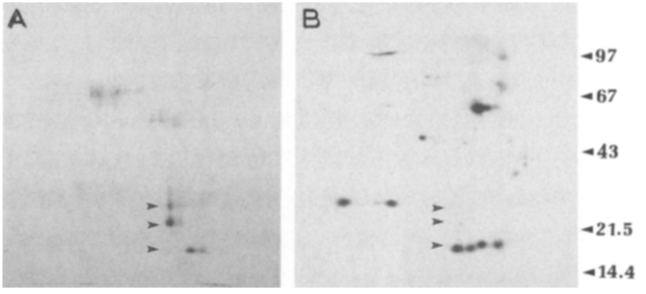


Figure 1. 2D SDS-PAGE of goat conceptus secretory proteins. Fluorograms of [^{35}S]-methionine-labeled culture medium proteins from day 17 goat conceptuses incubated in the absence (A) or presence (B) of 10 μg/ml of tunicamycin. cTP-1 isotypes are indicated by arrowheads. Molecular weight (x 10^{-3}) is indicated in the right hand margin. Gels are oriented with the acidic end to the right. Arrowheads indicate cTP-1.

macromolecules solubilized from tissue (Table 1). Tunicamycin did not affect incorporation of radioactivity into secretory proteins from medium (Table 1).

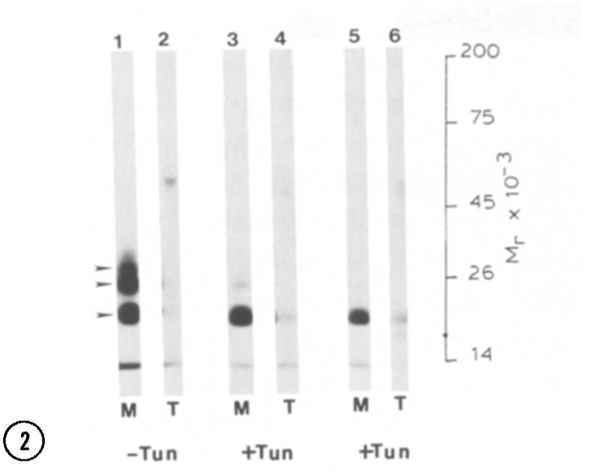
Polypeptides with molecular weights of 23,000, 21,000 and 17,000 were immunoprecipitated from medium with antiserum to bTP-1 (Fig. 2, Lane 1) and oTP-1 (not shown). Tissue contained far less cTP-1 than medium (Figure 2, Lanes 2,4,6). Addition of 5 or 10 μg/ml of tunicamycin to medium during culture resulted in an apparent increase in the electrophoretic mobility of the two larger species to a molecular weight of 17,000 (Fig. 2, Lanes 3,5). Quantification of cTP-1 immunoprecipitates demonstrated a decrease in the amount of the two larger polypeptide groups recovered from medium in response to tunicamycin (Table 1). There was no significant increase due to tunicamycin in the amount of the M_r=17,000 species found in medium (Table 1).

Application of [^{35}S]-methionine-labeled culture medium protein to a column of ConA-Sepharose resulted in 78% of cTP-1 not interacting with the lectin (Fig. 3A). SDS-PAGE analysis indicated that the M_r=17,000 polypeptides did not bind (Figure 3A). However, one-fifth of the total cTP-1 applied

Table 1. Effect of tunicamycin on protein and cTP-1 synthesis in vitro

	[^{35}S]cpm/embryo[a,c]		cTP-1[b,c]		
Tunicamycin	Tissue	Medium	23K	21K	17K
none	3.71	1.02	2.02**	6.11*	7.01
5 μg/ml	1.26*	1.00	0.08**	0.45*	6.04
10 μg/ml	1.36*	0.92	0.16**	0.44*	7.40
(±S.E.M.)	(0.55)	(0.20)	(0.29)	(1.24)	(2.27)

[a] Acid precipitable radioactivity (x 10^{-7}, tissue; x10^{-6}, medium).
[b] Integrated absorbance of fluorograms of cTP-1 immunoprecipitates from medium using antiserum to bTP-1.
[c] n=4 conceptuses (day 17) per treatment.
* P<0.02 compared to no tunicamycin treatment in same column.
** P<0.002 compared to no tunicamycin treatment in same column.



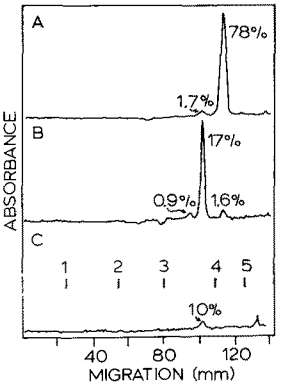


Figure 2. Immunoprecipitation of cTP-1. Fluorograms of [^{35}S]-methionine-labeled cTP-1 immunoprecipitated with antiserum to bTP-1 from conceptus medium (M) and tissue (T). Cultures contained tunicamycin at concentrations of 0 (-Tun, Lanes 1,2), 5 (+Tun, Lanes 3,4), or 10 μg/ml (+Tun, Lanes 5,6).

Figure 3. Interaction of cTP-1 with ConA-Sepharose. Densitometric scans of SDS-PAGE fluorograms of cTP-1 immunoprecipitated from ConA-Sepharose eluant fractions. A: unbound fraction; B: 10 mM α-methylglucoside eluate; C: 100 mM α-methylmannoside eluate. The percentage of cTP-1 in each peak from the total sample is indicated. Molecular weight standards are indicated: 1, phosphorylase b (97,000); 2, bovine albumin (66,700); 3, ovalbumin (43,000); 4, soybean trypsin inhibitor (21,500) and 5, ribonuclease A (14,400).

eluted with a molecular weight of 21,000 by using 10 mM α-methylglucoside (Figure 3B). Only a minor portion of the M_r=23,000 polypeptides eluted with either α-methylglucoside (Fig. 3B) or α-methylmmannoside (Fig. 3C).

Discussion

The results presented here suggested that cTP-1 is a mixture of glycosylated and nonglycosylated forms. Glycosylation was restricted to isotypes in the M_r=23,000 and M_r=21,000 groups. Tunicamycin, an inhibitor of N-linked glycosylation, reduced the mass of the two higher molecular weight classes of cTP-1 to 17,000, equivalent to the lowest molecular weight class. The M_r=21,000 class interacted with ConA and eluted under conditions selective for complex-type oligosaccharides (17). High mannose or hybrid types of N-linked oligosaccharides were not in abundance since very little cTP-1 eluted from ConA-Sepharose with α-methylmannoside. The M_r=17,000 group was not glycosylated with N-asparaginyl oligosaccharide since its electrophoretic mobility was not altered by tunicamycin and it did not bind to ConA. It must be cautioned that only N-linkage type of glycosylation was investigated in this report. O-linked oligosaccharides have been described on subtypes of human (21) and mouse (22) interferon-α. From the experiments reported here,

it was not possible to deduce the number of oligosaccharide chains on glycosylated forms of cTP-1.

oTP-1 is a group of four isotypes (pI 5.4 to 5.7) having a common molecular weight of 17,000 which do not contain oligosaccharide chains (13). bTP-1 has molecular weight classes of 24,000 and 22,000, each with multiple isotypes, which are glycosylated with N-linked oligosaccharides (15). The higher molecular weight class bears complex chains while the lower class has high mannose chains (15). No O-linked chains were detected on bTP-1 (15). bTP-1 contains one potential site for N-asparaginyl-oligosaccharide (Asn-Thr-Thr; residues 78-80) which is missing in oTP-1 (3). Multiple cDNA clones, varying slightly in the nucleotide sequence of the mature protein coding region, have been isolated for oTP-1 (3-6) and bTP-1 (3). In vitro translation assays suggested that the multiple oTP-1 mRNAs expressed account for the presence of oTP-1 isotypes (13). Tunicamycin reduced the number of apparent cTP-1 isotypes from six to four. The two isotypes in the 23,000 and the 21,000 classes shared common isoelectric points (approximately 6.0 and 5.7) which were clearly distinguishable from the two isotypes in the 17,000 class (pI of 5.5 and 5.2) following tunicamycin treatment. This suggested that cTP-1 charge heterogeneity was not due to differences in glycosylation. Thus, at least four cTP-1 mRNAs may exist.

In summary, cTP-1 was found to consist of glycosylated isotypes of M_r=23,000 and 21,000 and nonglycosylated isotypes of M_r=17,000. The function of carbohydrate on embryonic interferon during early pregnancy is unknown.

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