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Stage-Specific Alteration of Membrane Glycoproteins from the Rabbit Uterine Tract Epithelium

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ABSTRACT

Glycoproteins (GPs), proteins with covalently bonded carbohydrate complexes, are of prime interest because they are important components of cell surfaces (1,2). We have evaluated changes in GPs for three different reproductive stages of rabbits: estrus (non-ovulating), day-6 pseudopregnant (ovulating but not artificially inseminated) and day-6 pregnant (ovulating and artificially inseminated). The GPs were isolated and separated by one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis [1D SDS-PAGE] and then identified by alternate staining with a protein and a carbohydrate specific stain. The GPs were eluted out of the gels using electroelution followed by digestion with peptide-N-glycosidase F (PNGase F) enzyme. Eight epithelial GPs, with apparent molecular weights of 91, 70, 60, 55, 45, 28, 17, and 15 kD were detected in all three reproductive states. Relative proportions of four of the eight GPs (91, 70, 55, and 28 kD) significantly increased from estrus to day-6 of pregnancy or pseudopregnancy. There were no significant differences between pregnancy and pseudopregnancy implying little effect of the embryo on expression of maternal epithelial glycoproteins. Only two GP concentrations (45 kD and 17 kD) remained constant. Presence of carbohydrate on selected glycoproteins was confirmed by enzymic digestion.

Key Words: endometrium, glycoproteins, PNGase F
*Reprint requests

INTRODUCTION

A successful pregnancy is dependent on the implantation process once fertilization has occured. The luminal epithelium undergoes extensive differentiation in rabbits prior to the time of blastocyst implantation. This includes rapid cell proliferation and fusion of adjacent cells (3). However, investigation into stage-specific proteins of the endometrial epithelium has been limited. Lampelo et al. (1985) described stage specific proteins with apparent molecular weights 38,000, 55,000 and 84,000 using purified plasma membranes isolated from receptive rabbit endometrial scrapings (4). It was uncertain whether those proteins were components of epithelial or stromal membranes.

Glycoproteins (GPs), proteins with covalently bonded carbohydrate complexes, are of special interest because they are found to be components of the cell surface (1,2,5,6). Anderson et al. (1986) isolated two major glycoproteins with apparent molecular weights of 24,000 and 42,000 from the luminal epithelium of estrous, pseudopregnant and pregnant rabbit uterine tracts. The studies, using fluoroscein isothiocyanate-conjugated (FITC) lectins, suggested the presence of a glycoprotein with a N-sialic acid-D-galactose terminus in the rabbit endometrial epithelium (7). While the biological functions of these stage specific glycoproteins are not known, these glycoproteins are thought to be molecules of recognition/adhesion involved in cellular interactions between the trophoblast and the uterus during the initial stages of implantation.

The mechanisms by which the pre-implantation embryo and the maternal system of mammals recognize each other are poorly understood. Insufficient biochemical research has been done to find out the chemical signals which coordinate the selection of the embryonic implantation site. Inappropriate selection of embryonic implantation site could result in ectopic pregnancies where the developing embryo implants on an unusual site other than the uterus. In humans, ectopic pregnancies accounts for 0.5-1% of chemically recognized pregnancies (8). The ectopic pregnancies are often life threatening for the mother and require surgical intervention. GPs found in the maternal-embryo interface could play an important role in determining where the embryo implants. Therefore we wished to extend Anderson's work. The aim of this study was to develop a practical method to separate the GPs from total protein using an electroelution technique. Some isolated GPs were then subjected to enzymatic degradation to remove the intact carbohydrate for further chemical characterization. From these experiments, changes in types or concentration of one or more glycoproteins or oligosaccharides were observed. The data suggest specific functions of the glycoproteins or oligosaccharides in the process of embryo-maternal molecular recognition.

MATERIALS AND METHODS

Bovine serum albumin (BSA) (fraction V), phenylmethylsulfonyl flouride (PMSF), pepstatin A and Schiff reagent were purchased from Sigma Chemical Company (St. Louis, MO). Leupeptin was bought from Calbiochem Corporation (La Jolla, CA). Pepstatin A and Peptide-N-glycosidase F (PNGase F) [from *Flavobacterium meningosepticum*] were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The Sun Visage Image Analyzer was purchased from Microsystems, Inc., (Mountain View, CA) and programs for the image analyzer (densitometer) were obtained from BioImage Corporation (Ann Arbor, MI). All other chemicals were obtained from standard suppliers. All water used was doubly deionized and subjected to a millipore filter system.

Rabbit uterine tissues were obtained from Dr. M.J.K. Harper and were shipped on dry ice from the University of Texas Health Science Center at San Antonio, TX. Tissues were stored at –80°C until used. Mature, New Zealand White-Cambridge estrous females had been injected with 0.5 international units (IU) of porcine follicle stimulating hormone (FSH), subcutaneously, twice a day for 3 days. The rabbits were then artificially inseminated with sperm from fertile bucks and designated as pregnant (superovulated)

rabbits. After insemination, the animals were then injected intravenously with 100 IU of human chorionic gonadotropin (hCG) to induce ovulation. This was designated as zero time (9). The rabbits were then sacrificed (overdose of sodium pentabarbital, iv) at 144 hours (day-6) after zero time. All pregnancies were confirmed by gently flushing embryos from the uterus using Tyrode's buffer. For pseudopregnant (superovulated) rabbits, only the injections of FSH and hCG were given, as described above. Rabbits were then sacrificed at 144 hours (day-6) after zero time. No embryos were found in these animals. Rabbits not given the injections were considered to be estrus.

The uterine tissues were thawed, rinsed with ice-cold 0.9% (w/v) NaCl (saline) and weighed separately. The major procedure used to isolate luminal epithelial membrane proteins followed the intraluminal detergent solubilization method reported by Anderson et al. (7). Protein concentrations were determined following the method by Bradford (10) using bovine serum albumin (BSA) as a standard. The acetone precipitated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a slab gel by a modification of the method of Laemmli (11). Discontinuous gels, having a 9% to 15% horizontal step gradient in the resolving gel and a 4.5% stacking gel (7) were loaded with equal amounts of proteins (50μg for the protein stain and 100μg for the carbohydrate stain) in each lane. Separation was achieved at room temperature with a constant power of 15 Watts per gel, with two slab gels running simultaneously. After electrophoresis was completed one gel (with 50ug protein) was stained with coomassie brilliant blue (12) and the other (with 100μg protein) with the periodic acid-Schiffs (PAS) reagent (13). Glycoproteins were identified as those bands which appeared on both gels at the same location. With each SDS-PAGE performed, standards of known molecular weights (Bio-Rad Laboratories, Richmond, CA) and concentrations were also evaluated. The apparent molecular weight of each unknown glycoprotein was then calculated from the migration pattern of the standard proteins using a standard curve. Percentage area of each glycoprotein of the total glycoproteins detected in each reproductive state was evaluated using a Sun Image Analyzer, which evaluates the areas of each glycoprotein band relative to an intensity calibration curve. The SDS-PAGE gels stained by coomassie blue were used for this purpose. The integrated area of each glycoprotein was divided by the total area for all the glycoproteins detected in each reproductive state and multiplied by 100 to calculate the percentage. The gel stained with coomassie brilliant blue was then cut into sections to isolate the glycoproteins selected for further analysis. The sections were put into individual dialysis bags and placed in the electroelution chamber (Elutrap) containing 25mM Tris, 192mM Glycine (pH 8.3) and 0.1% (w/v) SDS. The glycoproteins were eluted at 200V applied between the two electrodes, for 8 hours (14). After elution, the solutions were removed, lyophilized and then stored at –70°C until needed.

Electroeluted and lyophilized glycoproteins were resuspended in 500mM Tris-HCl (pH 6.8) and divided into two fractions. One fraction was used for testing on SDS-PAGE and the second for enzymatic digestion. The second fraction was divided into two aliquots and each aliquot was lyophilized. One resulting pellet was resuspended in water (100μl) and placed in a solution containing 100mM ammonium bicarbonate (pH 7.2), 1%(v/v) Triton X-100 and incubated at 37°C for 24 hours with 2 units of peptide-N-glycosidase F (PNG-ase F) enzyme. Fetuin, a well studied glycoprotein from fetal calf serum, was used as a standard glycoprotein in development of the enzymatic digestion procedure. After

digestion of the glycoproteins with PNG-ase F results were compared by SDS-PAGE, with the non digested GPs. Changes in MW with and without digestion were recorded.

Statistics were performed using linear regression, analysis of variance (ANOVA), and Fisher's least significant difference (LSD) as applicable to determine the significant difference ($p < 0.05$) between reproductive states. Also mean and standard errors of the mean (SEM) were calculated for all data.

RESULTS

Apparent molecular weights of select glycoproteins were determined by simultaneous staining of two identical gels with coomassie blue or periodic acid Schiff's reagent after SDS-PAGE. Figure 1 represents the coomassie blue stained gel for proteins after SDS-PAGE. The protein bands stained with coomassie blue represents the proteins obtained from the rabbit uterine tract epithelium. These bands were matched with those bands obtained from periodic acid Schiff's stain for carbohydrates. The proteins stained with both the stains were identified as glycoproteins. A total of eight glycoproteins were detected in all three reproductive states (estrus, day-6 pseudopregnant and day-6

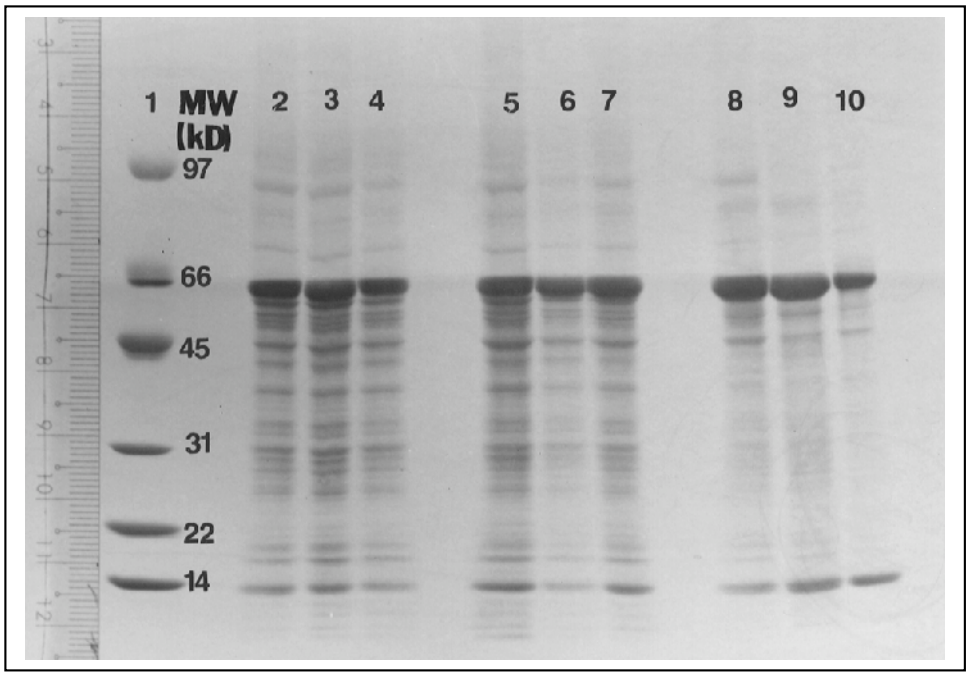

Figure 1. Coomassie Blue Stained SDS-PAGE Gels of Proteins Extracted from Rabbit Uterine Tissue. Protein stained gels of luminal epithelial membrane proteins from day-6 pregnant (lanes 2-4), day-6 pseudopregnant (5-7) and estrous (8-10) rabbit uteri. Protein standards (phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) are shown in lane 1. The values indicate the molecular weights (kD) of the standards used.

pregnant) by the two staining methods. However the band intensities were different in different reproductive states suggesting differences in relative abundance. Protein stained gels of luminal epithelial membrane proteins from day-6 pregnant (lanes 2-4), day-6 pseudopregnant (5-7) and estrous (8-10) rabbit uteri. Protein standards (phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) are shown in lane 1. The values indicate the molecular weights (kD) of the standards used. The apparent molecular weights of uterine GPs were estimated relative to the standards. The relative proportions, therefore, the relative concentrations of the glycoproteins, were evaluated from the areas of the glycoproteins detected by the image analyzer (Table 1). There were a total of eight glycoproteins present in the three reproductive states (estrous, pseudopregnant and pregnant). Of these, the image analyzer integrated only four (60, 45,17, and 15 kD) in the estrous reproductive state. The other four proteins were detected only visually but could not be integrated due to low intensity. The day-6 pregnant and pseudopregnant tissues had significantly higher (about ten fold) amounts of 91, 70, 55 and 28 kD glycoproteins compared to the estrous tissue. The estrous tissue had a significantly higher (two fold) concentration of the 60 kD and 15 kD glycoproteins compared to the pseudopregnant and pregnant tissues. There was no significant difference in percentage between the 45 kD and the 17 kD glycoprotein amounts among the three reproductive states. The 60 kD glycoprotein was the most predominant glycoprotein in all three stages. An overall significant increase in the glycoprotein amounts of molecular weights 91, 70, 55, and 28 kD was observed from estrous to pseudopregnant to pregnant tissue. Also, there was a significant decrease in the 60 kD and 15 kD glycoprotein from estrous to pseudopregnant and pregnant tissue. There were no significant differences in any of the glycoproteins in day-6 pregnant tissue relative to day-6 pseudopregnant tissue.

Table 1. Mean ± SEM of percentage intensity of each glycoprotein band of the total glycoprotein detected by the image analyzer.

Apparent molecular weight (kD)	Estrus % Glycoprotein	Day-6 Pseudopregnant % Glycoprotein	Day-6 Pregnant % Glycoprotein
91	*	13.3 ± 1.97^{a}	14.6 ± 1.45^{a}
70	*	9.48 ± 0.683^{b}	11.0 ± 0.993^{b}
60	46.4 ± 5.11^{c}	30.4 ± 3.51^{d}	24.9 ± 1.16^{d}
55	*	8.46 ± 0.0250^{e}	8.51 ± 0.430^{e}
45	12.9 ± 2.05^{f}	12.0 ± 0.828^{f}	8.94 ± 1.21^{f}
28	*	9.68 ± 0.106^{g}	10.1 ± 1.13^{g}
17	9.36 ± 2.10^{h}	8.43 ± 0.652^{h}	8.45 ± 0.603^{h}
15	24.6 ± 4.63^{i}	12.4 ± 0.074^{j}	13.6 ± 1.42^{j}

* Indicates bands present but below evaluation limits of the image analyzer.
Values with the same letter are not significantly different between reproductive states at $p < 0.05$ (ANOVA and Fisher's LSD).

Figure 2 represents the image analyzer data in the form of a bar graph. The Y axis represents the percentage intensity of total (intensity detected by the image analyzer is integrated as A x mm^2) for the glycoproteins ± standard error of the mean (SEM) detected by the image analyzer. The X axis represents the apparent molecular weights(kD) of the glycoproteins detected. The SEM is indicated on the top of each bar. The SEM values were relatively small (0.30% to 2.2%) compared to the mean values. Three rabbit uterine GPs of molecular weight 60, 45, and 15 kD were digested with PNGase F and electrophoretic behavior changes were compared to the well known glycoprotein, fetuin. A loss of 7kD was obtained for each of the three rabbit uterine GPs studied. If we assume the average MW of a monosaccharide is 212 then a 7kD would be 30-33 sugar residues attached to these GPs. The loss of approximately 50% of the MW of the 15 kD glycoprotein and only about 10% of the MW of the 60 kD glycoprotein is interesting. The lower molecular weight loss of the rabbit uterine tract glycoproteins compared to fetuin could be due to two factors. The rabbit uterine glycoproteins could be mostly O-linked, or the rabbit uterine GPs have less glycosylations than fetuin. Under the same experimental conditions, fetuin lost about 21 kD of molecular weight (data not shown). The undigested fetuin had a higher apparent molecular weight by SDS-PAGE than the digested fetuin as predicted. Multiple bands in both the digested and the undigested fetuin were observed and are due to the fact that fetuin is composed of several

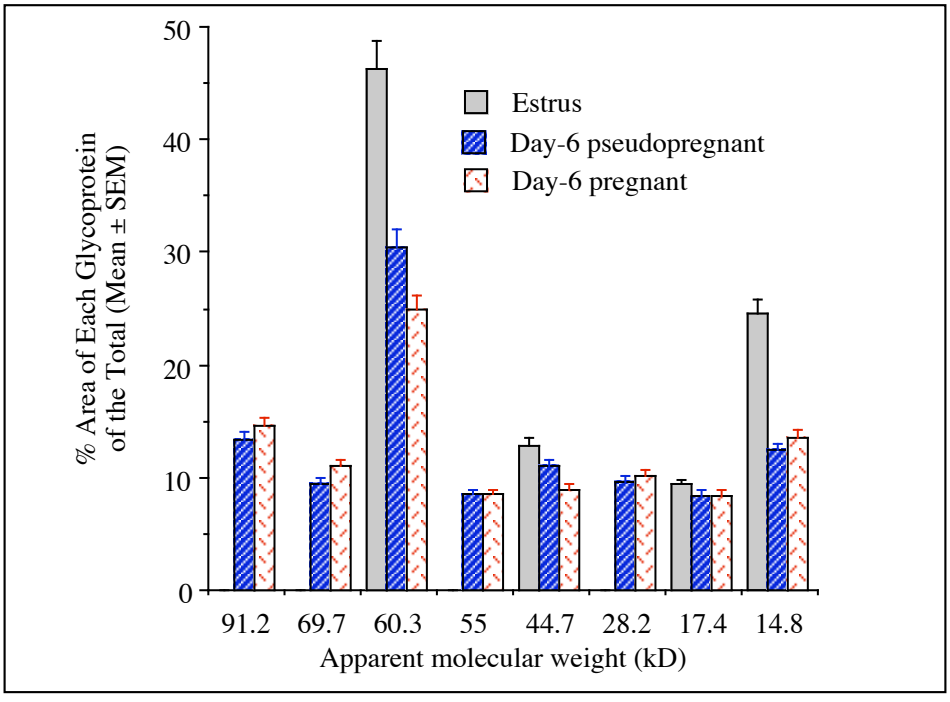

Figure 2. Image Analyzer Data of Glycoproteins after SDS-PAGE. The mean ± SEM of the % of intensity of each GP of the total GP is shown for the 8 GPs detected. For all stages the 60 kD protein had the highest value.

glycopeptides of fairly close molecular weights (15). Also fetuin has both O-linked and N-linked oligosaccharides. The PNGase enzyme is specific for N-linked glycoproteins and, therefore, would hydrolyse only the N-glycosidic bonds.

DISCUSSION

The method of solubilization used in our study for the plasma membrane from rabbit uterine tract by intraluminal incubation with detergent was a novel technique reported by Anderson et al.(7). This technique offers advantages over the previous method adopted by Lampelo et al. (4) which used endothelial scrapings of the uterine tissue. With the technique by Anderson and co workers the apical membrane of luminally-exposed cells can be partially solubilized, leaving the apical junctional complexes, as well as the lateral and basal regions of the plasma membranes intact. This provides specificity for the surface cells that the blastocyst would encounter, eliminating contamination by cryptal, glandular, stromal and vascular membranes (7). The detergent solution contained an adequate concentration of protease inhibitors (pepstatin A, leupeptin and PMSF) to avoid band broadening in SDS-PAGE. From these studies, then, it is obvious that the selection of isolation method is clearly important.

A total of eight glycoproteins were detected in all three reproductive states (estrous, day-6 pseudopregnant and day-6 pregnant) after SDS-PAGE, but the band intensities were different in different reproductive states. The number of protein bands detected by coomassie blue staining increased from estrous to pseudopregnant and pregnant tissue. This increase was more evident for the smaller molecular weight proteins than for the larger molecular weight proteins. The larger number of protein bands in pseudopregnant and pregnant tissue could be attributed to an increase in uterine endometrial synthesis of proteins after fertilization. All six standards were observed with good separation by SDS-PAGE in our study using the discontinuous gradient gels, so we consider these gradient gels to be a very useful method to separate proteins (the correlation coefficient for the log MW vs. protein mobility was 0.990). However, estimation of glycoprotein molecular weights must be reviewed with caution since it has been reported that glycoproteins can migrate differently than non glycosylated proteins of the same molecular weights (16).

The glycoprotein relative proportions obtained from the image analyser were different for each of the three reproductive states. The day-6 pseudopregnant and the day-6 pregnant tissue had significantly higher (approximately ten fold) amounts of 91, 70, 55,28 kD glycoproteins than the estrous tissue. The estrous tissue had a significantly higher amount of the 60 kD (one and a half to two fold) and the 15 kD (almost two fold) glycoproteins compared to the day-6 pseudopregnant and the day-6 pregnant tissue. There was no significant difference between the proportions of 45 kD and 17kD glycoproteins in any of the three reproductive states. Therefore, it is clear that of the total eight glycoproteins present in the rabbit uterine tract luminal epithelium, the majority (four) significantly increase in amounts from estrous to pseudopregnancy or pregnancy. Only two glycoproteins (45 kD and 17 kD) remain the same. The increase in glycoprotein amounts

can be attributed to the remodelling of the uterine epithelium directed by the ovarian steroid hormones, since there was no apparent quantitative difference of glycoproteins concentrations between the pseudopregnant and pregnant tissue. The presence of the embryo aparently has little effect on uterine glycoprotein content. This correlated with the reports by Anderson et al. (1986) who also report blastocysts are not required for stage specific glycoproteins (7). The 45 kD glycoprotein detected in our study could be comparable to the 42kD glycoprotein detected in Anderson's study. Lampelo et al. (1985) detected three proteins (84kD, 55kD and 38kD) from the receptive stages and two proteins from the non-receptive stages (78kD and 30kD) of the rabbit endometrial scrapings (4). Unfortunately the MW values of these two studies do not correspond.

The glycoprotein with an apparent molecular weight of 15kD evident throughout each of the three reproductive stages corresponds well with the apparent molecular weight of uteroglobin (15kD), a pregnancy specific rabbit uterine protein (17,18). The fact that the relative proportion of this glycoprotein decreased in day-6 pseudopregnant (12.4 ± 0.074 %) and the day-6 pregnant tissue (13.6 ± 1.42 %) relative to estrous (24.6 ± 4.63 %) by almost one half correlates well with the findings of Beier (19). He reported that uteroglobin reaches a maximum concentration on day-3 to day-4 after ovulation. The overall results considering the 15 kD glycoprotein confirms hormonal control which stimulates the production of this glycoprotein in the uterine endometrium in preparation for the implanting blastocyst.

Whyte and Heap (1989) reported a 17kD molecular weight glycoprotein from the uterine endometrium of pregnant ewes which is similar to our MW of 17 kD found in all three stages tested. They have also reported glycoproteins from pregnant ewes with molecular weights of 45 kD and 50-60 kD and 80 kD which correspond to some of the glycoproteins (45 kD and 55 kD) detected by this study (20). This suggests there are similarities between glycoproteins present within reproductive tracts of different mammalian species. It also implies that the functions of these proteins could be comparable. However Tachi and Tachi (1979) suggest that there is a potential for species specificity involved in the blastocyst implantation to the maternal tissue (21). This specificity could be either a result of different types or concentrations of glycoproteins present in the lumen, or a different mechanism other than a glycoprotein mediated one. Studies by Fazleabas et al. (1985) have indicated a purple, iron-containing glycoprotein known as uteroferrin (Uf) with an apparent molecular weight of 35 kD, synthesized by the glandular epithelial cells of the uterine endometrium of pigs (22). Gel filtration has revealed a second, less abundant form of Uf which is pink in color with a molecular weight of approximately 80 kD. Studies by Murray et al.(1989) indicated another Uf-associated glycoprotein of molecular weight 40-50 kD from the pig uterine tract endometrium (23). We did not detect any proteins with purple or pink colors in the unstained gels. However, the absence of these proteins could be due the fact that the rabbits are taxonomically less related to pig or the rabbit Uf is present in a much lower concentration. More work remains to be done in this area.

A hormone-dependent endometrial protein designated "progestagen-associated (or dependent) endometrial protein " (PEP) is a glycoprotein detected from the human endometrium with a molecular weight of 47 kD (24). PEP has an apparent molecular weight close to the 45 kD glycoprotein detected in our studies. The slightly different

molecular weight observed could be due to the conformational variations of the PEP or due to glycosylation of this protein (either the number or the types of linkages of the oligosaccharide). However, since this rabbit protein does not change as a function of the reproductive state and presumbly the human one does, the functions of these two proteins may not be comparable.

From these data, therefore, it is possible to conclude that variations in types and concentrations of glycoproteins occur relative to the reproductive state. Due to the experimental procedure of obtaining these glycoproteins, they are most likely epithelial in origin. The data also clearly show that all glycoproteins do not appear to change in the same manner. This demonstrates that there is some specificity in the regulation of protein synthesis in the rabbit reproductive system and that the specificity is under hormonal control. The data imply that modifications in the maternal glycosylation of uterine epithelial cells will markedly affect pre-implantation embryo-maternal interactions.

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