Lactosaminoglycan Assembly, Cell Surface Expression, and Release by Mouse Uterine Epithelial Cells*

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The kinetics of assembly, cell surface expression, secretion, and degradation of the major lactosaminoglycan (LAG)-bearing glycoproteins in mouse uterine epithelial cells have been studied. LAGs have been shown previously to be synthesized preferentially by these cells in the uterus and are expressed at the cell surface, where they participate in cell adhesion processes (Dutt, A., Tang, J.-P., and Carson, D. D. (1987) Dev. Biol. 119, 27-37). We utilized selection on pokeweed mitogen-Sepharose, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequent electroelution to isolate the major LAG-bearing glycoproteins. The intact LAG-bearing glycoproteins exhibited very high apparent M, (>500,000) both by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and molecular exclusion chromatography under dissociative conditions. The subset of LAGs at the cell surface exhibited a half-life of approximately 11 h, whereas total cell-associated LAGs had a half-life of 6 ± 1 h. Pulse-chase experiments indicated that the transit time of LAG core proteins from the rough endoplasmic reticulum to the site of LAG addition in the Golgi was 30-45 min. LAG glycoprotein transit from the Golgi to the cell surface required at least an additional 30-45 min. The major metabolic fate of the cell-associated LAGs was secretion to the medium with no evidence of lysosomal degradation. Some (30%) of the LAGs appeared to be released to the medium via the action of cell surface proteases. Epithelial cell surfaces bound fluoresceinlabeled pokeweed mitogen, indicating the constitutive presence of LAG-bearing molecules at the cell surface; pokeweed mitogen binding to the cell surface was completely blocked by 10 μM chitotriose. These observations provide the first comprehensive description of the intracellular transport and metabolism of this interesting class of glycoproteins of the uterine epithelial cell surface.

The differentiative events in the adult uterus are unique in that they are primarily dependent upon the levels of the steroid hormones estrogen and progesterone. The major uterine morphogenetic events include embryo implantation and decidualization in the pregnant uterus and the cyclical endometrial changes during the proliferative proestrus phase of the nonpregnant uterus. Because glycoproteins have been implicated in cell recognition and adhesion phenomena during morphogenesis in a wide variety of systems (1), our laboratory has been involved in delineating the roles of glycoconjugates in the various cellular interactions of the mouse uterus and in determining how the expression of these molecules is regulated by estrogen and progesterone, i.e. the same hormones controlling important uterine biological functions.

Lactosaminoglycans (LAGs) compose a distinct class of large, complex polysaccharides containing a repeat Gal-61 + branching structures with glucosamine residues, at the luminal surface of the epithelium (12). Nonetheless, relatively little is known about the kinetics of their assembly or metabolic fate in any system. Uterine LAGs appear to be primarily N-linked, highly branched structures with M, values ranging from 4,000 to 15,000 (13). These structures are resistant to a variety of chemical and enzymatic treatments that degrade glycosaminoglycans. Recently, we have demonstrated that estrogen stimulates LAG synthesis in mouse uteri in marked preference to other N-linked and O-linked glycoconjugates (13); moreover, LAGs appear to be synthesized preferentially by the epithelial cells of the uterus (11). Hormone-dependent structural modifications of LAG oligosaccharide chains also have been observed to take place in mouse uterine epithelium (14). It is interesting that the uterus, one of the few adult tissues undergoing hormone-dependent differentiation and development,
ment, also expresses LAGs in a hormone-dependent manner. In order to characterize further these distinctive uterine glycoproteins, in the present studies we focused on the isolation of the major LAG-bearing glycoproteins and on aspects of their assembly, intracellular transit, cell surface expression, and subsequent metabolic fate in mouse uterine epithelial cells.

**EXPERIMENTAL PROCEDURES**

**CF-1 female mice**, the source of uterine tissue, were purchased from Harlan Sprague-Dawley (Houston, TX). Tissue culture media components were obtained from Irvine Scientific (Santa Ana, CA) and Flow Laboratories Inc. (McLean, VA). *[^H]Heparin* (0.5 mCi/ml) was from Du Pont-New England Nuclear. Amplify and [*[^35S]methionine* (1,200 Ci/mmol) were obtained from Amersham Corp.; and [*[^3H]glucosamine* (30 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). Octyl glucoside, Sephadex G-50 (fine), Pronase, fluorescein isothiocyanate-pokeweed mitogen, pokeweed mitogen-Sepharose, heparin, heparan sulfate, hyaluronic acid, dextran (molecular weight, 9,000), blue dextran (molecular weight > 2 X 10^6), dextran (molecular weight, 71,000), o-lactalbumin, tunicamycin, chloroquine, cycloheximide, leupeptin, benzamidine, aprotonin, chymostatin, pepstatin, antipain, trypsin, CHAPS, and FITC were all purchased from Sigma. Tyrode-ethylicel was from Eastman Kodak. Di-O-acetylchitotriose was obtained from Boehringer Mannheim. Guanidine hydrochloride was purchased from Schwarz/Mann. Endo-7-galactosidase (Pseudoomonas keratanase) and keratan sulfate were obtained from Miles Laboratories. Pronase was also obtained from Miles. Guanidine hydrochloride was from Boehringer Mannheim. Guanidine hydrochloride was from Schwarz/Mann. Endo-7-galactosidase (Pseudo- monas keratanase) and keratan sulfate were obtained from Miles Scientific (Naperville, IL). Endo-7-galactosidase (Escherichia freundii) was purchased from V-Labs (Covington, LA). N,N',N"-Triacetelrhamnose was from E-Y Labs (San Mateo, CA). All chemicals used were reagent grade or better.

**Cell Cultures and Metabolic Labeling**—Primary cultures of epithelial cells were prepared from excised uteri of CF-1 mice as described previously (11). Metabolic labeling with [*[^3H]glucosamine* (100 mCi/ml) was performed in serum-free medium as described previously (11); labeling with [*[^35S]methionine* (500 mCi/ml) was performed in a low methionine (10 mM) serum-free medium (Eagle's modified medium with Earle's salts and supplemented with 10 mM L-glutamine) containing 50 units/ml penicillin and 50 µg/ml streptomycin sulfate/ml for 16-18 h at 37 °C in a humidified atmosphere of air and 5% carbon dioxide, 95:5 (v/v).

**Cell Surface Radioiodination**—Primary cultures of uterine epithelia were prepared as described above and then rinsed several times with ice-cold phosphate-buffered saline (PBS) and maintained on ice. Cell surface radioiodination was performed as described before (15). Briefly, 1 ml of ice-cold PBS containing 25 µg/ml lactoperoxidase and 1 mCi NaI was added to the cell layer. Ten microliters of a solution of 0.125% (w/v) hydrogen peroxide containing 2 M guanidine was added to the cells. The cell layer was rinsed at least five times with an ice-cold solution of PBS containing 10 mM NaCl and then extracted as described below except that 1 mM NaCl was included in all the buffers.

**Preparation of Cell Extracts and Isolation of LAG Glycoproteins**—Radiolabeled cells were extracted for 1-2 h with a dissociating buffer consisting of PBS containing 6 M urea, 0.1% (v/v) octyl glucoside, and protease inhibitors (16) at 25 °C. The extract was desalted on a 0.8 X 20-cm Sephadex G-50 gel filtration column equilibrated with PBS containing 0.2 M urea and 0.1% octyl glucoside. The LAG glycoproteins were selected from the void volume fractions by binding them to pokeweed mitogen-Sepharose in the same buffer containing protease inhibitors. The bound LAG glycoproteins were removed from the lectin-Sepharose either by elution with 10 mM N,N',N"-triacetelrhamnose or by boiling in SDS-gel sample buffer for 3 min.

**Equilibrium and Pulse-Chase Labeling Studies**—For equilibrium labeling experiments, epithelial cells were labeled with [*[^3H]glucosamine* overnight as described above. After being rinsed several times with serum-free unlabeled medium, the cells were incubated in this “chase” medium for time intervals up to 24 h. The medium, constituting the secreted fraction, was collected at each time point and analyzed for LAGs as described below. After rinsing with serum-free medium, the cells were incubated in serum-free medium containing 50 µg/ml Pronase for 30 min on ice. These conditions have been shown previously to be optimal for release of cell surface LAGs (11). This treatment produced no evident cell detachment, and subsequent viability has been shown previously to be >90% by trypan blue exclusion (11). The medium collected at the end of this incubation constituted the Pronase-released, “cell surface” fraction, which was analyzed subsequently for LAGs. The residual cell layer was collected in PBS with a rubber policeman, sonicated, and then analyzed for LAGs; this material constituted the “intracellular” fraction. In other pulse-chase experiments, short pulses, i.e. 15 min, were employed, and chase times were extended to 90 min.

**Analyses of LAGs**—The secreted and cell surface fractions were chromatographed on a 1 X 30-cm column of Superose 12 (Pharmacia LKB Biotechnology Inc.) in a Beckman Instruments system eluted with 2 M guanidine HCl, 20 mM Tris acetate (pH 7.0), and 0.01% (w/v) octyl glucoside as described previously (17). The radioactive macromolecules eluting in the void volume of the column were pooled and analyzed for endo-7-galactosidase sensitivity and binding to pokeweed mitogen-Sepharose as described (13). The intracellular fraction was subjected to β-elimination, trichloroacetic acid precipitation, Pronase digestion, and Sephadex G-50 chromatography as described previously (16, 18); the chromatographed material migrating at the void volume also was analyzed as above for LAG content by endo-7-galactosidase sensitivity and pokeweed mitogen binding exactly as described previously (15). In some cases, material in the cell surface and secreted fractions were subjected to β-elimination followed by trichloroacetic acid precipitation to remove O-linked oligosaccharides. Typically, less than 10% of the radioactivity was removed by this procedure. The acid-insoluble material then was digested extensively with Pronase for further analyses of glycopeptides. The elution times that bound material was applied subsequently to either wheat germ lectin-agarose or D. stramonium lectin agarose. The material was applied in PBS batch wise at room temperature with constant rotary agitation. The wheat germ lectin-agarose then was eluted for 1 h at room temperature with constant rotary agitation with PBS containing 0.1 M N-acetylglucosamine to remove non-LAG components (19). Both lectin resins were then finally eluted with 4 M guanidine HCl, 1% (w/v) CHAPS, 20 mM Tris acetate (pH 7.0) to obtain the bound material. Digestions with E. freudii endo-7-galactosidase were performed at room temperature in 50 mM sodium acetate (pH 5.8) containing 0.3 units of enzyme/ml. After 24 h, an additional 0.3 unit/ml enzyme was added and the incubation continued for 24 h more. The products of this digestion were analyzed by Sephadex G-50 chromatography (1 X 30 cm) equilibrated with 0.1 M ammonium bicarbonate.

**Pokeweed Mitogen Binding to Cell Surfaces**—Cell monolayers cultured on 4-well (11 X 16-mm) multiwell culture dishes (Nunc) were fixed at room temperature for 15 min with 1% (v/v) formalin in PBS. After being rinsed several times with PBS containing 0.1% (w/v) bovine serum albumin, the cells were incubated in this same buffer containing 2 µg/ml fluorescein isothiocyanate-streptavidine-conjugated pokeweed mitogen either with or without 10 mM chitotriose at room temperature for 45 min in the dark. The cells were rinsed gently several times with PBS containing 0.1% (w/v) bovine serum albumin and subsequently visualized with an inverted microscope equipped with epifluorescence. Micrographs were taken at a magnification of 200 X. Other Procedures—SDDS-PAGE was carried out under reducing conditions as described (20). Electrophoresis of LAG glycoprotein from gel fragments was performed using a Bio-Rad model 422 electrophoreser according to the manufacturer's specifications. Chemical deglycosylation of electrophoresed LAG glycoproteins by trifluoromethanesulfonic acid was performed as described (21) except that the deglycosylation was extended to 24 h. Fluorography of [*[^3H]glucosamine* and [*[^35S]methionine* labeled samples was performed after the fixed gel was incubated in the fluorescent enhancer Amplify.

**RESULTS**

**Isolation of Pokeweed Mitogen-binding LAG Glycoproteins**—Epithelial LAG-bearing glycoproteins were selected from other glycoproteins using the LAG-specific lectin pokeweed mitogen (4, 13). The lectin-selected fractions then were analyzed by SDS-PAGE. Cells were labeled either metabolically with [*[^3H]glucosamine* or [*[^35S]methionine* or vectorially at the cell surface with NaI (21). Fig. 1, lanes A and D, shows the profiles of total epithelial extracts labeled with NaI and [*[^35S]methionine*, respectively. Lane B shows that the pattern of pokeweed mitogen-Sepharose-bound, radiodinated proteins specifically eluted with N,N',N"-triacetelrhamnose was essentially the same as that obtained after boiling the lectin-Sepharose with SDS-PAGE sample buffer to accom-
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by SDS-PAGE, they both gave rise to material that stayed in the stacking gel and barely entered the running gel (data not shown). Consequently, both fractions were considered to contain the same components and, for simplicity, will be referred to as one in further studies.

We had performed a number of studies previously which focused on the oligosaccharide components of the LAG glycoproteins in mouse uteri as well as uterine epithelial cells (11, 13). These studies demonstrated that LAGs were N-linked and completely resistant to a variety of chemical and enzymatic treatments that degrade glycosaminoglycans. To extend these observations, we set out to study the protein cores of these molecules. To accomplish this, cells were labeled metabolically with \[^{35}S\]methionine and selected by pokeweed mitogen affinity chromatography. This material was fractionated by SDS-PAGE and subjected to autoradiography. The \[^{35}S\]methionine-labeled band corresponding to the \[^{3}H\]glucosamine-labeled LAG glycoproteins at the top of running gel and the corresponding stacking gel slice were electroeluted separately and used further. Although the LAG oligosaccharides are N-linked structures (11, 13), we were unable to release them from their protein cores with peptide:N-glycanase under a variety of conditions in which internal N-linked glycoprotein standards were completely digested (data not shown). Consequently, we relied on chemical deglycosylation (21) to release LAGs and any other oligosaccharides linked to the protein cores.

We have shown previously that a 24-h deglycosylation time is required to shift the intact LAG glycoproteins from a form that barely enters a running gel on SDS-PAGE to a form that enters the running gel (22). As shown in Figs. 1 and 3, \[^{35}S\]methionine-labeled LAG glycoproteins appeared to be very large by both SDS-PAGE and molecular exclusion chromatography under dissociative conditions. In contrast, the LAG glycoproteins eluted as a broad peak exhibiting hydrodynamic radii similar to that of oligosaccharides of 10,000-40,000 molecular weight (see Fig. 4). The chromatographic profile of the intact \[^{35}S\]methionine-labeled LAG glycoproteins on Superose 12 was very similar to that of the \[^{3}H\]glucosamine-labeled cell surface LAG glycoproteins as seen in Fig. 3B, i.e. they eluted at the void volume; however, after chemical deglycosylation with trifluoromethanesulfonic acid both the core peptides derived from intact LAG glycoproteins and Pronase digests of LAG glycoproteins were converted completely to forms that migrated in the Superose 12 gel filtration column with hydrodynamic radii similar to that of proteins of 8,000 molecular weight (Fig. 2). When the core peptides were analyzed by SDS-PAGE, they appeared as a diffuse band ranging from 4,000 to 5,000 M, (data not shown). When the deglycosylated core peptides were subjected to Pronase digestion and chromatographed under identical conditions, they were totally converted to lower molecular weight species migrating near the fully included column volume. Thus, it appeared that the oligosaccharide chains of LAG glycoproteins protected the protein cores from proteolytic attack, and once deglycosylated, these core proteins were susceptible to proteolytic digestion.

To determine if the extensive incubation time required to deglycosylate fully the LAG glycoproteins caused protein degradation, we performed similar digestions on ovalbumin. When subjected to a 24-h deglycosylation, ovalbumin was also quantitatively degraded to lower M, peptides. These data demonstrated that peptide bond breakage was very likely to have occurred during the deglycosylation step for the LAG glycoproteins as well. Although this procedure generated only one class of core peptides of similar M, and hydrodynamic

Fig. 1. SDS-PAGE of pokeweed mitogen-selected glycoproteins. LAG glycoproteins were prepared as described under "Experimental Procedures" and subjected to SDS-PAGE of the following acrylamide concentrations: lanes A–E, 7.5–10% (w/v) gradient; lane F, 10% (w/v). Lanes A–C contain proteins labeled vectorially with \[^{14}C\], lanes D and E contain proteins labeled metabolically with \[^{35}S\]methionine, and lane F contains \[^{3}H\]glucosamine-labeled proteins. Lanes B and E contain pokeweed mitogen-binding protein specifically eluted with N-A-acetylglucosaminidase; lanes C and F contain lectin-bound proteins extracted from the resin with SDS-PAGE sample buffer for 3 min at 100°C. The arrow to the right of the figure indicates the M, of the major lectin-bound glycoproteins.

plish elution (lane C). Therefore, the latter procedure was adopted as a convenient routine method in subsequent studies. Many lectin-selected, \[^{35}S\]methionine-labeled proteins were observed which distributed over a broad range of molecular weights (lane E); however, \[^{3}H\]glucosamine-labeled pokeweed mitogen-binding glycoproteins migrated primarily as very high M, (>200,000) components that either remained in the stacking gel or barely entered the running gel (lane F). This low electrophoretic mobility was also observed when 8 M urea was included in the gel system (data not shown). Moreover, low electrophoretic mobility was observed for the \[^{125}I\]-labeled, pokeweed mitogen-binding proteins of the cell surface (lanes B and C). SDS-PAGE on 10% gels (lane F) better resolved the \[^{3}H\]glucosamine-labeled, lectin-selected components, whereas gradient gels were used to resolve the more complex profiles obtained in lanes A–E. The \[^{3}H\]glucosamine-labeled LAG glycoproteins that entered the running gel migrated similarly to the \[^{35}S\]methionine- or \[^{125}I\]-labeled LAG glycoproteins that entered the running gel on gradient gels (data not shown). In all cases, most of the lectin-selected radioactivity remained dispersed throughout the stacking gel.

The \[^{3}H\]glucosamine-labeled material in the lectin-selected fraction has been shown previously to be primarily composed of LAG-containing oligosaccharides (13). Since more bands appeared in the \[^{35}S\]methionine- and \[^{125}I\]-labeled lectin-selected fractions than in the \[^{3}H\]glucosamine-labeled lectin-selected fractions, it appeared that lectin selection alone was insufficient to isolate the intact \[^{35}S\]methionine-labeled LAG glycoproteins. Consequently, we used a combination of lectin selection and electroelution of material either in the stacking gel or that barely entering the running gel from SDS-PAGE.
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Secreted LAG glycoproteins and LAG glycopeptides released from the cell surface appear to be very large in size (Fig. 3, A and B). No reduction in size was observed on preincubation with either 8 M urea at room temperature overnight or 50 mM dithiothreitol at 90 °C for 1 h (data not shown). The Pronase glycopeptides derived from LAG glycoproteins eluted near the void volume of a Sephadex G-50 column; however, these glycopeptides proved to be fairly heterogeneous in size and displayed hydrodynamic radii similar to those of oligosaccharides of 5,000-20,000 molecular weight by Superose 12 gel filtration chromatography (Fig. 4).

It was noted that the LAG glycopeptides isolated from the secreted fraction appeared to lack much of the higher Mr forms served in the cell-associated forms. It was important to avoid freezing of the LAG glycopeptide fractions during such analyses because this led to an irreversible aggregation. This not only caused the glycopeptides to migrate at the void volume of molecular exclusion columns but also reduced the interaction of the LAGs with lectins and decreased their susceptibility to endo-β-galactosidase (data not shown).

Analysis of [3H]Glucosamine-labeled LAGs in Different Cellular Fractions—It was most convenient to use [3H]glucosamine-labeled material to study LAG metabolism since this material is enriched for LAG glycoproteins. Consequently, procedures were developed to detect LAGs in various fractions derived from epithelial cells. Cell cultures were labeled with [3H]glucosamine and the secreted, cell surface, and intracellular fractions were isolated as described under "Experimental Procedures." Pronase glycopeptides were prepared from each fraction (see Fig. 4) and were analyzed for LAG content by various criteria, viz. endo-β-galactosidase sensitivity and lectin binding, two established diagnostic features of LAGs, in general. As shown in Table I, all three fractions exhibited similar levels of susceptibility to Pseudomonas endo-β-galactosidase, ranging from 6 to 9%, which is comparable to the 10 to 15% digestibility observed for total LAG from either epithelial cells in vitro (11) or whole uteri (13). A much greater proportion of each fraction was susceptible to E. freundii endo-β-galactosidase. Although the Pseudomonas enzyme releases a tetrasaccharide from these structures (13), the E. freundii enzyme released a heterogeneous mixture of lower Mr products ranging in size from 800 to 2,000 molecular weight (data not shown). Therefore, there appeared to be a variety of sites in these LAG structures which were susceptible to the E. freundii enzyme and were resistant to the Pseudomonas enzyme.

Uterine LAG glycopeptides are highly branched structures...
glycopeptides also were tested for their ability to bind to other fractions derived from two separate cell cultures. Averages and ranges of duplicate determinations performed on LAG with 0.1 binding as described under "Experimental Procedures." For lectin-labeled cells and chromatographed on a 1 X 30-cm column of Superose 12 under dissociative conditions as described under "Experimental Procedures." The migration positions of blue dextran (71K), [3H]heparin (12K), a 3,000 M, (3K), and [35S]O, (V) are indicated.

**TABLE I**

**Characteristics of lactosaminoglycan in various fractions**

Cells were labeled with [3H]glucosamine for 20-24 h. The resulting fractions were obtained by trichloroacetic acid precipitation of the β-eliminated fractions. Pronase glycopeptides were prepared from various [3H]glucosamine-labeled lactosaminoglycan fractions. These fractions were analyzed for endo-β-galactosidase sensitivity and lectin binding as described under "Experimental Procedures." For lectin-binding studies, the LAG fractions were fractionated by pokeweed mitogen-agarose into bound (PWM+) and unbound fractions. The PWM+ fractions were subsequently applied to either wheat germ lectin (WGA) or D. stramonium lectin (DSA). The percentage of PWM+, radioactivity remaining associated with the resin after elution with 0.1 M N-acetylglucosamine is presented as the strongly binding fraction (WGA; Ref. 19). The percentage of PWM, radioactivity that bound to D. stramonium lectin is presented. The values represent the averages and ranges of duplicate determinations performed on LAG fractions derived from two separate cell cultures.

| Lactosaminoglycan property | Lactosaminoglycan fractions |
|----------------------------|-----------------------------|
|                            | Secreted | Cell surface | "Intracellular" |
| Endo-β-galactosidase sensitivity |
| *Pseudomonas* | 8 | 9 | 6 |
| *E. freundii* | 95 ± 2 | 64 ± 4 | 31 ± 1 |
| Lectin binding |
| PWM | 94 ± 1 | 78 ± 1 | 75 ± 2 |
| PWM → WGA | 93 ± 1 | 87 ± 1 | 87 ± 1 |
| PWM → DSA | 84 ± 4 | 68 ± 5 | 83 ± 2 |

that bind well to pokeweed mitogen-Sepharose. Binding to this lectin can be improved if the oligosaccharides are released from the peptide by hydrazinolysis (13). All three epithelial cell LAG glycopeptide subfractions bound well to pokeweed mitogen-Sepharose (Table I). The pokeweed mitogen-binding glycopeptides also were tested for their ability to bind to other LAG-binding lectins, namely, *D. stramonium* lectin (3) and wheat germ lectin under stringent conditions (19). In all cases, a very large fraction of the pokeweed mitogen-binding glycopeptides also bound well to other LAG-binding lectins. Collectively, these criteria indicated a substantial enrichment for LAG-containing molecules in all three of the [3H]glucosamine-labeled subfractions, i.e. the secreted fraction, the Pronase-released cell surface fraction, and the remaining cell-associated intracellular fraction of uterine epithelial cells. These approaches were utilized to monitor the intracellular and extracellular distribution of LAGs.

**Metabolic Half-life of Epithelial LAGs**—Cell cultures were labeled with [3H]glucosamine as described under "Experimental Procedures," and the secreted and cell-associate fractions were analyzed for LAGs after 6, 2, 4, 10, and 24 h of chase in unlabeled medium. As shown in Fig. 5A, after being stable for the initial 2 h, LAGs were lost gradually from the cell-associated fraction, reaching a lower limit of approximately 20% of the zero time values by 10 h. This level did not decrease further even after 24 h. The t1/2 of loss from the cell-associated fraction ranged from 5 to 7 h in three experiments. The decrease in LAGs in the cell-associated fraction could be accounted for almost completely by secretion since secreted LAG accumulation in the medium was coordinated with the loss from the cell-associated fraction.

Having established the turnover rate of total cell-associated LAGs, we next investigated the turnover rate of the cell surface LAGs since this subset of molecules is the one implicated in cell adhesion functions (11) and was therefore of particular interest. Pronase-released cell surface fractions were analyzed for LAGs at each time point. As shown in Fig. 5B, it appeared that the cell surface LAGs were more stable than bulk cell-associated LAGs, exhibiting a t1/2 of approximately 11 h. Even after 24 h, 33% of the initial level of cell surface LAGs remained. It was concluded that LAG-bearing glycoproteins apparently were more stable at the cell surface.

![Fig. 4. Superose 12 chromatography of Pronase glycopeptides of various LAG glycoprotein fractions](image)

**Fig. 4.** Superose 12 chromatography of Pronase glycopeptides of various LAG glycoprotein fractions. Pronase glycopeptides were prepared from either the cell surface (panel A), intracellular (panel B), or secreted (panel C) LAG fractions of [3H]glucosamine-labeled cells and chromatographed on a 1 X 30-cm column of Superose 12 under dissociative conditions as described under "Experimental Procedures." The migration positions of blue dextran (71K), [3H]heparin (12K), a 3,000 M, (3K), and [35S]O, (V) are indicated.

![Fig. 5. Turnover rate of epithelial LAGs](image)

**Fig. 5.** Turnover rate of epithelial LAGs. Cultured cells were labeled to equilibrium overnight with [3H]glucosamine and chased for up to 24 h as described under "Experimental Procedures." Panel A depicts the kinetics of LAG secretion (●—●) and loss from the total cell-associated pool (○—○). Panel B shows the kinetics of loss of radioactivity from the cell surface LAGs over this time period.
than in the total cell-associated fraction.

The pulse-chase studies data (Fig. 5A) indicated that almost all of the LAGs lost from the cell were secreted into the medium. In order to test this possibility further and to determine whether epithelial LAGs undergo intracellular, e.g. lysosomal degradation, we treated cultures with chloroquine during the chase period to inhibit lysosomal degradative processes. As shown in Table II, chloroquine had only a slight stimulatory effect on LAG secretion in contrast to epithelial heparan sulfate proteoglycans whose secretion is enhanced 2.5-fold under similar conditions (17). Furthermore, LAGs did not accumulate in the cell-associated fraction in chloroquine-treated cells (data not shown), which supports the notion that the metabolic fate of most LAGs is secretion or release from the cell surface. Proteolytic release from the cell surface has been observed in the case of epithelial heparan sulfate proteoglycans (17). To determine whether the release of the LAGs to the medium might be protease mediated, we included a protease inhibitor mixture (PIM) in the medium during the chase. This resulted in a 27% inhibition of secretion after 7 h, suggesting that proteolytic cleavage was partially responsible for the loss of LAG from the cell surface.

When EDTA, either alone or in combination with PIM, was added to the chase medium, there was a similar decrease (39 and 31%, respectively) in the level of secretion. These observations suggested the involvement of divalent cation-dependent proteases in LAG release. Collectively, these experiments suggest that plasma membrane association and subsequent protease-mediated release is one pathway by which LAG is released from cell surfaces.

Intracellular Transport of LAGs—The kinetics of transit of LAGs to the cell surface from intracellular sites of biosynthesis were examined. Since the formation of complex carbohydrates, including LAGs, is completed in the Golgi (23), we wished to determine the minimum time of LAG transit from the Golgi to the cell surface. Cell cultures were pulsed with [3H]glucosamine for 15 min, the minimum time required to generate detectable amounts of [3H]glucosamine-labeled LAG, and rapidly washed. The arrival of radiolabeled LAGs at the cell surface was monitored by accessibility to externally added proteases. As shown in Fig. 6, LAG arrival at the cell surface is first detectable 25 min (by extrapolation) after initiation of the chase period. Radiolabeled LAG continued to accumulate at the cell surface throughout the chase period examined, i.e. up to 90 min. Considering that the pulse time extended over 15 min, we estimated the minimum transit time for LAGs to reach the cell surface from the Golgi complex to be between 25 and 40 min, i.e. 33 ± 7 min.

In a separate experiment, we estimated the maximum time required for LAGs to travel from the rough endoplasmic reticulum to the Golgi, i.e. from the site of core protein synthesis to the site at which terminal steps of complex N-linked carbohydrate chain formation (in this case, LAG assembly) take place. The time required for LAG synthesis to cease in response to an inhibitor of protein synthesis was used to estimate this transit time. Preliminary studies demonstrated that protein synthesis was inhibited by more than 95% within 5 min of cycloheximide addition (data not shown).

Cells were labeled to equilibrium overnight with [3H]glucosamine and then chased for 7 h in the presence of the indicated agent. LAG released to the medium then was determined as described under "Experimental Procedures." The concentrations of protease inhibitor mixtures (PIMs) and chloroquine used were as described previously (17); the level of Na2EDTA was 10 mM. The numbers represent the average of values obtained from duplicate determinations in a representative experiment. The numbers in parentheses show the ratio of the average experimental value to the average control value.

| Addition       | LAG secreted dpm |
|----------------|------------------|
| Control        | 103,000 ± 14,000 (1.00) |
| Chloroquine    | 130,000 ± 22,000 (1.26) |
| PIMs + EDTA    | 75,000 ± 5,000 (0.73) |
| EDTA           | 65,000 ± 7,000 (0.61) |
| PIMs + EDTA    | 72,000 ± 8,000 (0.69) |
FIG. 8. Cell surface binding of pokeweed mitogen. Primary cultures of epithelial cells were prepared and processed for staining with fluoresceinated pokeweed mitogen as described under "Experimental Procedures." Panels B and D show fluorescein isothiocyanate-pokeweed mitogen binding to cells plated at high and low densities, respectively; panel F shows inhibition of binding in the presence of 10 mM chitotriose. Panels A, C, and E show the corresponding phase-contrast images. Magnification, × 200.

minimum transit time to the plasma membrane from the Golgi was similar, i.e. 33 ± 7 min. Therefore, LAG glycoproteins required approximately 1 h to reach the cell surface from the rough endoplasmic reticulum.

Cell Surface Binding of Pokeweed Mitogen—Fluoresceinated pokeweed mitogen was used to detect LAGS at uterine epithelial cell surfaces. Primary cultures of epithelial cells were prepared, fixed, and incubated with fluorescein isothiocyanate-pokeweed mitogen in the absence and presence of 10 mM chitotriose. As shown in Fig. 8, positive diffuse staining occurred on the surfaces of all cells plated at a high density, although to varying degrees (panel B). This variation was attributed partially to the cells being in different planes of focus and partially to LAGs being distributed at inaccessible basolateral cell surfaces. When the cells were plated at a lower density, almost all displayed diffuse pokeweed mitogen binding at the surface (panel D). The more homogeneous patterns of binding are apparently due to the enhanced accessibility of the fluoresceinated molecule to the more exposed cell surfaces. Lectin binding was completely blocked by the inclusion of 10 mM chitotriose in the incubation medium (panel E), suggesting a requirement for the N-acetylgalactosamine residues for lectin binding. These data are compatible with the earlier observation that LAGs comprise greater than 90% of the [3H] glucosamine-labeled glycoconjugates that can be released from the cell surfaces with proteases (11). These experiments visually demonstrated the constitutive cell surface distribution of epithelial LAGs.
DISCUSSION

The surface localization of LAG oligosaccharides has been established in a variety of systems including hematopoietic cells, macrophages, lymphocytes, thyroid, kidney, uterus, and developing embryos (2, 8, 24–28). LAGs are especially interesting in such systems since they exhibit changes in levels or structure, or both, as a function of differentiation, development, and transformation. Well characterized extracellular matrix molecules such as laminin and placental fibronectin also are glycosylated with LAGs under certain conditions (29, 30), and LAG-bearing molecules are present in extracellular matrices and cell contact sites and on apical aspects of epithelia under certain conditions (14, 27, 28). LAGs are a major group of cell surface glycoproteins on uterine epithelial cells and appear to participate in adhesion processes that take place between these cells (11). It was shown recently that monoclonal antibodies recognizing fucosylated LAG structures bind to the apical surfaces of mouse uterine luminal and glandular epithelia as well as to luminal secretions during the peri-implantation period (31). In the present studies, we have used vectorial labeling, accessibility to externally added proteases, and staining of intact epithelial cells with a LAG-specific lectin to demonstrate cell surface distribution of epithelial cell LAG-bearing glycoproteins. Furthermore, for the first time, the kinetics of intracellular movement of LAGS from their sites of biosynthesis to their surface location and subsequent metabolic fate have been described. A model summarizing the data is shown in Fig. 9. The minimum intracellular transit time of 30 min for LAGs from the rough endoplasmic reticulum to the Golgi (Fig. 9, step 1) is comparable to that of the heparan sulfate proteoglycans of these cells (17). However, whereas proteoglycans take only 4–8 min to reach the cell surface from the Golgi, LAGs take much longer, i.e. 33 ± 7 min (Fig. 9, step 3). This difference probably reflects a lack of association of these two surface components en route to the cell surface. The turnover rates of LAGs and heparan sulfate proteoglycans of these cells differ markedly as well, being 6 and 2 h, respectively. The different intracellular transit times and turnover rates of heparan sulfate proteoglycans and LAGs also indicate that the cell surface is not synthesized and turned over en masse in these cells since these molecular species undergo distinct metabolic fates. It has been shown that different membrane and secreted proteins travel from the Golgi to the cell surface at different rates in human hepatoma Hep G2 cells (32). In these studies, the rough endoplasmic reticulum to Golgi transit time was variable and appeared to be the rate-limiting step. In contrast, the Golgi to plasma membrane transit time was rapid and similar for all proteins studied, all of which were glycosylated with conventional, low $M_r$, N-linked chains. Consequently, it is possible that the large differences in the Golgi to cell surface transit time between epithelial cell heparan sulfate proteoglycans and LAG glycoproteins reflect their extensive differential glycosylation with high $M_r$ oligosaccharide chains.

Epithelial LAG glycoproteins exhibit very large apparent $M_r$, both by molecular exclusion chromatography under disequilibrium conditions and by SDS-PAGE. The corresponding limit glycopeptides, prepared by exhaustive Pronase digestion, displayed apparent $M_r$ values ranging from 5,000 to 20,000. Previous studies have shown that the LAG oligosaccharides released by hydrazinolysis display molecular weights ranging from 4,000 to 15,000 (13). Thus, it is possible that multiple LAG chains are present on the same protein core. In fact, in the present studies, we found that the presence of LAG chains was able to protect segments of protein cores from proteolytic attack. It was important to avoid freezing LAG glycoproteins or glycopeptides during their analyses since this led to an irreversible aggregation. As a result, the aggregates became more resistant to endo-$eta$-galactosidase and, generally, bound less well to lectins. We have been unable to reduce the size of these aggregates with guanidine, urea, sulfhydryl reagents, or a variety of detergents. Therefore, the exact nature of this aggregation remains unclear. Nonetheless, it must be considered that the apparently huge $M_r$ of the intact LAG glycoproteins may be due, in part, to aggregation.

The LAG oligosaccharides are very resistant to enzymatic and chemical removal. Although these oligosaccharides could be removed by chemical deglycosylation procedures, the extended incubation times required to remove the resistant oligosaccharides are likely to have caused some peptide bond breakage. Previously, we have found that the LAG oligosaccharides were resistant to other chemical depolymerization techniques apparently due to their high content of hexosamine (13). Although a relatively simple pattern of peptides was generated after chemical deglycosylation, we cannot be sure whether there is one or several LAG-bearing core proteins. In any event, Pronase-digested LAG glycopeptides contain peptides of at least 5 kDa which can be further reduced in size by proteases if the LAG chains are removed. Consequently, LAG oligosaccharides can protect their protein carriers from proteolytic attack. The high $M_r$, aggregative characteristics along with the tendency of other cell surface components to copurify the LAG glycoproteins are consistent with the proposed role for these molecules in cell adhesion functions (11); however, these properties further complicate the process of isolating individual LAG glycoproteins.

The subset of cell surface LAGs displayed metabolic characteristics distinct from the total cell-associated LAG population, e.g. a slower turnover rate. This apparent stability reflects, in part, the result of an equilibrium between arrival of preformed intracellular LAG glycoproteins at the cell surface and their release to the medium. Release to the medium
may partially reflect a normal secretory function of these cells (Fig. 9, step 2). In addition, cell surface-exposed LAGs are released by endogenous, divalent cation-dependent proteases (Fig. 9, step 4). Even though a complex mixture of protease inhibitors was used in these studies, we cannot be sure that cell surface proteolytic activity was completely inhibited. Thus, it remains possible that all LAGs are released from the cell surface by the action of cell surface proteases. If multiple processes are used to release LAG glycoproteins from these cells, we could account in part for the selective stability of the cell surface-associated LAG population. For example, it may be that proteases release the cell surface-retained forms of LAG glycoproteins, whereas other LAGs are more rapidly lost by direct secretion or other cell surface release processes, e.g., hydrolysis of glycosphosphatidylinositol anchors (33). Proteolytic release from the cell surface has been suggested for other cell surface glycoproteins, i.e., heparan sulfate proteoglycans (34), including those of uterine epithelial cells (17). Consequently, this system may be of general utility in modifying the surface composition of these cells.

A very large fraction (80-90%) of epithelial LAGs was destined for the cell surface or secretion; no evidence of lysosomal degradation was obtained (Fig. 9, step 5). This does not mean that LAG-bearing proteins may not reside in lysosomes, as has been shown by Carlsson et al. (35). Indeed, the intracellular LAGs described in this study had certain characteristics, e.g., size distribution and sensitivity to endo-

β-galactosidase, that were distinct from cell surface and secreted/released LAGs. Moreover, approximately 20% of the cell-associated LAGs were retained in the cell layer after extended chase periods, approximately half of these were inaccessible to externally added Protease and so appeared to be intracellular. Although they may be distinct from LAG glycoproteins destined for the cell surface or release, we do not know if these intracellular LAGs are in lysosomes. In addition to the present studies, secretion of structurally related, LAG-containing glycoproteins by peri-implantation murine, ovine, and bovine embryos and polarized rat uterine epithelial cells has been reported (31, 36-38). These observations appear to be in sharp contrast to the suggestion that membrane anchoring of the protein is a feature common to LAG-bearing glycoproteins, whereas secretory proteins are restricted to being glycosylated with typical asparagine-linked complex and hybrid-type chains (39). Clearly, this generalization does not hold for the epithelial LAG-bearing glycoproteins; however, it must be considered that since a large fraction of these molecules is proteolytically released from the cell surface (Fig. 9, step 4), secreted LAG glycoproteins may be derived from membrane-bound forms.

The uterus is one of the few adult tissues that undergoes steroid hormone-dependent differentiation. LAG synthesis has been shown to be stimulated dramatically by estrogen (13) in this system. Recent evidence from our laboratory shows that immature mouse produce O-linked LAG glycoproteins, and supplementation with estrogen alone is not sufficient to restore the ability of the immature uterus to switch LAG expression to be of the N-linked type (40). It is possible that both estrogen and progesterone are involved in modulating epithelial surface LAGs, either with regard to total levels, protein carriers, and/or fine oligosaccharide structure, to control their adhesive properties during the estrous cycle. Other uterine epithelial cell glycoproteins have been shown to be metabolized more rapidly in response to estrogen (41). Thus, LAG glycoprotein metabolism also may be hormonally regulated. Hormone-dependent alterations in turnover rates could serve as a means of reducing cell surface LAG concentrations when necessary. In addition, it must be considered that LAG glycoproteins may be distributed among the apical versus basolateral aspects of these epithelial cells in response to steroid hormones. Consequently, polarized epithelial cells in culture, which appear to be steroid hormone responsive (42), would be an excellent experimental system for further studies of the expression of this interesting class of epithelial cell glycoconjugates.

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