Increased LAMP-2 Polylactosamine Glycosylation Is Associated with Its Slower Golgi Transit During Establishment of a Polarized MDCK Epithelial Monolayer

Ivan R. Nabi*† and Enrique Rodriguez-Boulan‡

*Département de pathologie, Université de Montréal, Montréal, Québec, Canada H3C 3J7; and ‡Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021

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An endogenous Madin-Darby canine kidney (MDCK) lysosomal membrane glycoprotein that exhibits a basolateral targeting pathway to the lysosome is shown here to exhibit significant N-terminal amino acid sequence identity to lysosomal associated membrane proteins (LAMP-2) of other species. During establishment of the MDCK monolayer after only 1 d of culture, this canine LAMP-2 has a larger molecular size (110 kDa) than following formation of a confluent monolayer after 3 d of culture (100 kDa) due to the increased presence of N-linked polylactosamine oligosaccharide chains. Neither polylactosamine glycosylation of LAMP-2 in MDCK cells nor truncation of N-linked oligosaccharide chains of LAMP-2 in a ricin-resistant MDCK-RCA^R cell line influenced the basolateral polarity of its targeting. However, the rate of basolateral delivery of LAMP-2 in MDCK cells plated for 3 d was significantly faster ($t_{1/2} = 28$ min) than in 1-d cells ($t_{1/2} = 40$ min); in MDCK-RCA^R cells the rate of basolateral delivery at both 1 and 3 d of plating was similar ($t_{1/2} = 40$ min). The rate differential in MDCK cells occurred after arrival of LAMP-2 to the Golgi apparatus because the rate of acquisition of endoglycosidase H resistance was the same ($t_{1/2} = 25$ min) at both days of plating. The rate of transit of LAMP-2 through the Golgi apparatus to the basolateral domain was therefore far more rapid (~ 4 -fold) in 3 d compared with 1-d MDCK cultures. The increased polylactosamine glycosylation of MDCK LAMP-2 at early times of plating during the establishment of a confluent epithelial monolayer may thus be related to its longer residence time in the Golgi apparatus.

INTRODUCTION

The major glycoproteins of the lysosome are a class of heavily glycosylated transmembrane proteins of 100–120 kDa referred to alternately as LAMPs (lysosomal associated membrane proteins), lgps (lysosomal glycoproteins), LEP (lysosome endosome protein), or LIMP (lysosomal integral membrane protein) (reviewed in Kornfeld and Mellman, 1989; Fukuda, 1991). Human lamp-1 and lamp-2 were initially isolated as the major cellular proteins carrying the polylactosamine (repeated Gal β 1-4GlcNAc β 1-3) glycosylation motif (Carlsson *et al.*, 1988), which is found on only certain LAMP gly-

cosylation sites (Carlsson and Fukuda, 1990). Changes in expression and structure of polylactosamine glycans are associated with cellular differentiation and oncogenesis (Fukuda, 1985). Extensive polylactosamine glycosylation is found on the LAMP-1 homologous P2B antigen of the highly metastatic cell line MDAY (Hefernan *et al.*, 1989), and increased polylactosamine glycosylation of LAMP-1 and LAMP-2 are associated with increased metastatic ability in sublines of human colon carcinoma (Saitoh *et al.*, 1992). Decreases in LAMP polylactosamines are found with endodermal differentiation of the embryonal carcinoma cell line F-9 with retinoic acid (Amos and Lotan, 1990) and differentiation of HL-60 cells to granulocytes with dimethyl sulfoxide (Lee *et al.*, 1990). A similar decrease over time in

† Corresponding author.

expression of polylectosamines was correlated with differentiation of the polarized intestinal epithelial cell line CaCo-2 (Youakim *et al.*, 1989).

The role of LAMPs and their extensive glycosylation is not known. The heavily glycosylated ectoplasmic domain of LAMP-1 and LAMP-2 protects LAMPs from protease degradation in the lysosome as indicated by the short lifetime of LAMPs in the presence of tunicamycin (Barriocanal *et al.*, 1986), and it has been suggested that the extensive glycosylation, including polylectosamines, of LAMPs serves to coat the lysosomal membrane protecting it from protease degradation (Carlsson *et al.*, 1988; Granger *et al.*, 1990). However, LAMPs are found not only in the lysosome but also within endosomes and on the cell surface (Lippincott-Schwartz and Fambrough, 1986, 1987; Griffiths *et al.*, 1988; Mane *et al.*, 1989; Amos and Lotan, 1990; Do *et al.*, 1990; Febbraio and Silverstein, 1990; Nabi *et al.*, 1991; Harter and Mellman, 1992; Saitoh *et al.*, 1992). In the polarized Madin-Darby canine kidney (MDCK) epithelial cell line, the biosynthetic pathway of an endogenous LAMP lysosomal glycoprotein to the lysosome was shown to be from the Trans-Golgi network (TGN) direct to the basolateral cell surface and/or early endosome and then via the endocytic pathway to the lysosome (Nabi *et al.*, 1991). Similarly, in nonpolarized cells a chimeric molecule containing the lysosome protease sensitive ectodomain and transmembrane domain of vesicular stomatitis virus G protein and cytoplasmic tail of LEP100 exhibited transient surface expression before delivery and degradation in lysosomes (Mathews *et al.*, 1992). LAMP surface expression is not solely due to the biosynthetic pathway as LAMPs can recycle at low levels between lysosomes, endosomes, and the cell surface, and more substantially in activated platelets and in cells treated with lysosomotropic agents (Lippincott-Schwartz and Fambrough, 1986, 1987; Mane *et al.*, 1989; Febbraio and Silverstein, 1990; Nabi *et al.*, 1991).

Polylectosamine oligosaccharide chains are the major carriers of glycosylation motifs recognized by a number of endogenous mammalian lectins including the 14 kDa, 34 kDa, and selectin types (Leffler and Barondes, 1986; Merkle and Cummings, 1988; Feizi, 1991; Sato and Hughes, 1992). That LAMPs, along with the extracellular matrix components fibronectin and laminin, are a major cellular carrier of polylectosamine glycosylation suggests that their surface expression is involved in lectin-mediated cell adhesion (Carlsson *et al.*, 1988; Sato and Hughes, 1992). Polylectosamines of laminin are recognized by both 14- and 31-kDa endogenous β -galactoside binding lectins, and lectin secretion regulates cellular binding to polylectosamines of laminin (Woo *et al.*, 1990; Zhou and Cummings, 1990; Cooper *et al.*, 1991; Ochieng *et al.*, 1992; Sato and Hughes, 1992). Developmentally regulated secretion of an L-14 β -galactoside lectin is responsible for myoblast detachment

from laminin before formation of tubular myofibers (Cooper *et al.*, 1991). In Chinese hamster ovary (CHO) cells the primary cell surface carrier of polylectosamine glycosylation is LAMP-1, which is recognized by a mammalian S-type lectin (Do *et al.*, 1990). The increased expression of LAMP-1 polylectosamines in retinoic acid treated F-9 cells is paralleled by the increased expression of a 14.5-kDa β -galactoside lectin (Lotan *et al.*, 1989; Amos, and Lotan, 1990). Altered expression of polylectosamines on LAMPs may be related to their involvement in lectin-mediated intercellular interactions at various stages of cellular differentiation and trans-formation.

We show that after only 1 d of culture, during establishment of the MDCK monolayer, an endogenous MDCK LAMP-2 exhibits increased polylectosamine glycosylation compared with cells plated for 3 d that have established a confluent epithelial monolayer. These results correspond to previous work demonstrating the decreased LAMP polylectosamine glycosylation with cellular differentiation in another epithelial cell line, the highly differentiated CaCo-2 intestinal epithelial cell line (Youakim *et al.*, 1989). To address the mechanisms regulating LAMP-2 polylectosamine glycosylation during acquisition of a differentiated epithelial phenotype, we have assessed the influence of polylectosamine glycosylation on the biosynthetic targeting pathway of this endogenous MDCK LAMP-2.

MATERIALS AND METHODS

Cells and Materials

MDCK II and ricin-resistant MDCK-RCA^R cells (Meiss *et al.*, 1982) were grown in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum, glutamine, and nonessential amino acids (GIBCO, Grand Island, NY) in an air-5% CO₂ atmosphere at constant humidity. When grown on filters, 2×10^6 cells were seeded on 24-mm Transwell polycarbonate filters (0.4 μ pore size) (CoStar, Cambridge, MA) and cultured for 1 or 3 d with the medium of the 3-d cultures changed on the second day after plating. All cultures routinely obtained a transepithelial resistance of $>240 \Omega/\text{cm}^2$ measured using a Millicell-ERS resistance system (Millipore, Bedford, MA). Ascites fluid from the mouse AC17 monoclonal antibody (mAb) was obtained as previously described (Nabi *et al.*, 1991). Lectin-conjugated beads, were purchased from E-Y Labs (San Mateo, CA). Except where indicated all chemicals were purchased from Sigma (St. Louis, MO).

N-terminal Sequencing

A membrane fraction was prepared from homogenized dog kidney by sequential centrifugation, lysed with 0.5% Triton X-100 in tris(hydroxymethyl)aminomethane pH 8, and the AC17 antigen isolated by immunoprecipitation with AC17 mAb conjugated to oxidized agarose beads by reductive amination (Stults *et al.*, 1989a). After washing the beads, the AC17 antigen was removed by boiling in 0.1 M ammonium acetate and concentrated by lyophilization. A sample was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to PVDF blotting paper (Millipore). The band corresponding to the AC17 antigen was cut out, and 20 N-terminal amino acids were sequenced by Edman degradation (Scott Germanos, Microchemistry Core Facility, Memorial-Sloan Kettering Cancer Center, New York, NY).

Figure 1. N-terminal sequence homology of the AC17 antigen to LAMP-2. Sequencing of 20 N-terminal amino acids of the AC17 antigen immunopurified from dog kidney by Edman degradation revealed significant sequence identity to human, mouse, and rat LAMP-2 (Fukuda *et al.*, 1988; Cha *et al.*, 1990; Granger *et al.*, 1990). Shaded boxes represent sequence homology of the AC17 antigen with LAMP-2 from other species and slashed boxes represent unidentifiable amino acids.

Metabolic Labeling, Immunoprecipitation, Surface Immunoprecipitation, and Glycosidase Digestions

Metabolic labeling with ^{35}S -methionine/cysteine (^{35}S -Express, New England Nuclear, Boston, MA), immunoprecipitation, endoglycosidase digestions, and surface immunoprecipitation of the AC17 antigen from MDCK and MDCK-RCA^R cells were exactly as previously described except that AC17 ascites fluid was used at a 1:200 dilution for immunoprecipitation and 1:100 for surface immunoprecipitation (Nabi *et al.*, 1991). Seven percent polyacrylamide gels were used to achieve the best separation of 1-d and 3-d LAMP-2, and 8% gels were used when both the full size protein (~100 kDa) and the core polypeptide (~40 kDa) were run on the same gel. Quantitation of autoradiograph bands was performed using a BioImage gel scanner (Dr. A. Herscovics, McGill Cancer Center, McGill University).

Lectin Precipitation

The lectins used were as follows: Concanavalin A (Con A), Erythrina cristagalli agglutinin (ECA), Lycopersicon esculentum agglutinin or tomato lectin (LEA), Ulex europaeus agglutinin-1 (UEA-1), leucoagglutinating Phaseolus vulgaris agglutinin (L-PHA), and Limulus polyphemus agglutinin or horseshoe crab lectin (LPA). Immunoprecipitated AC17 antigen was removed from Protein A Sepharose beads by boiling in 50 μl phosphate-buffered saline (PBS) containing 0.1% SDS, pooled, and equivalent amounts diluted in 1 ml PBS containing 0.2% NP-40 and 0.2% bovine serum albumin in which 50 μl of lectin-conjugated sepharose beads had been preincubated overnight in the cold. The AC17 antigen was incubated with the lectin bead mixture for 1 h in the cold, then washed three times with the above buffer and once with PBS before analysis by SDS-PAGE. For Con A and LPA the buffer was supplemented with 2 mM Ca^{2+} and Mg^{2+} .

RESULTS

Identity of the AC17 Antigen as Canine LAMP-2

A monoclonal antibody raised against MDCK cells recognized a lysosome specific glycoprotein, previously referred to as the AC17 antigen, that exhibited significant similarity to members of the LAMP family of lysosomal glycoproteins (Nabi *et al.*, 1991). As seen in Figure 1, the 20 N-terminal amino acids of the AC17 antigen exhibit significant sequence identity with human, rat, and mouse LAMP-2 (Fukuda *et al.*, 1988; Cha *et al.*, 1990; Granger *et al.*, 1990) suggesting that the AC17 antigen is the canine homologue of LAMP-2. Of 18 identifiable N-terminal amino acids, 15 were identical to those of human LAMP-2 and 11–12 to mouse lamp-2 and rat lgp110, respectively. The nonidentifiable amino acids in the AC17 sequence correspond to a cys-

teine (#13), which is not detectable by Edman degradation, and an asparagine (#4), which is located in an N-linked glycosylation sequence, Asn-X-Thr, whose glycosylation would render it undetectable (Kaplan *et al.*, 1987). The significant degree of homology of the N-terminal sequence of the AC17 antigen to LAMP-2 of other species suggests that it is the canine counterpart of LAMP-2 and will subsequently be referred to as MDCK LAMP-2.

Increased Polyglactosamine Glycosylation of LAMP-2 from 1-d MDCK Cultures

MDCK LAMP-2 isolated from cells plated on polycarbonate filters at confluence for 1 d exhibits a larger molecular size in polyacrylamide gels (110 kDa) compared with LAMP-2 from cells plated for 3 d (100 kDa). The increased size is a consequence of altered N-linked glycosylation because after endoglycosidase F digestion the core polypeptide has an identical size (Figure 2A). Although LAMP-2 has been shown to contain O-linked oligosaccharides (Carlsson *et al.*, 1988; Granger *et al.*, 1990), digestion with O-glycanase and neuraminidase in addition to endoglycosidase F did not alter the migration of the digestion product. The absence of a shift in mobility of LAMP-2 after O-glycanase digestion could be due to a number of reasons. Access of O-glycanase might be sterically hindered by the presence of

Figure 2. Increased polyglactosamine glycosylation of LAMP-2 in MDCK cells plated for 1 d. (A) LAMP-2 labeled overnight with ^{35}S -methionine/cysteine was immunoprecipitated from 1- or 3-d MDCK cells and then left undigested (-); digested with endoglycosidase F (EF); or a mixture of endoglycosidase F, O-glycanase, and neuraminidase (MIX). (B) LAMP-2 labeled overnight with ^{35}S -methionine/cysteine was immunoprecipitated from 1- and 3-d MDCK cells and then left undigested (-), digested with neuraminidase (NE), endo- β -galactosidase (E-gal), or endo- β -galactosidase and neuraminidase (NE + E-gal).

Figure 3. Lectin binding of LAMP-2 from 1- and 3-d MDCK cultures. LAMP-2 labeled overnight with ^{35}S -methionine/cysteine was immunoprecipitated from 1- or 3-d MDCK cells and either digested with neuraminidase (+NE) (bottom) or left undigested (top) and then reprecipitated with the indicated panel of six lectin-sepharose beads. The bands were quantitated by densitometry and the percentage binding relative to the largest value of the six lectins is indicated below the respective bands.

the numerous N-linked oligosaccharide chains of LAMP-2, the O-linked oligosaccharides of LAMP-2 may not be susceptible to O-glycanase digestion, or, alternatively, removal of O-linked oligosaccharides might not alter the mobility of the protein in SDS polyacrylamide gels. However, treatment of 1- and 3-d LAMP-2 with endo- β -galactosidase, specific for internal β -galactoside residues of polylactosamines (Fukuda *et al.*, 1978), reduced the increased size of 1-d LAMP-2 such that it comigrated with 3-d LAMP-2 (Figure 2B). Treatment with neuraminidase alone did not significantly alter the migration of either 1- or 3-d LAMP-2 indicating that the major change responsible for the altered mobility of LAMP-2 is the increased polylactosamine glycosylation of 1-d LAMP-2.

Confirmation of the increased expression of polylactosamines on 1-d LAMP-2 was obtained from lectin binding studies. Immunoprecipitated LAMP-2 was reprecipitated with agarose beads conjugated to the following lectins: ECA, Con A, UEA-1, L-PHA, LEA, LPA (Figure 3). From this panel of six lectins, LAMP-2 was bound to the largest extent by the galactose-specific lectin ECA. Binding of LAMP-2 to other lectins was normalized to ECA except for the case of 3-d LAMP-2 without neuraminidase treatment for which binding to the mannose-specific lectin Con A was slightly larger than to ECA. More 1-d LAMP-2 (59%) was recognized by the polylactosamine specific lectin L-PHA than 3-d LAMP-2 (41%). The differential L-PHA binding was enhanced after neuraminidase digestion such that essentially all 1-d LAMP-2 and only 55% of 3-d LAMP-2 were recognized. ECA exhibits specificity for the Gal β 1-4GlcNAc disaccharide component of polylactosamines (Lis *et al.*, 1985), and the differential recognition of 1- and 3-d LAMP-2 by ECA and L-PHA may reflect the increased specificity of L-PHA for the β 1-6 branching of polylactosamines from the mannose core of N-

linked oligosaccharides (Cummings and Kornfeld, 1982; Dennis *et al.*, 1987). No binding was detected to LEA that exhibits a specificity for elongated polylactosamine chains (Merkle and Cummings, 1987), suggesting that the polylactosamines of MDCK LAMP-2 are short (<3 Gal-GalNAc dimers). Binding of L-PHA has been shown to be influenced by the presence of sialic acid (Bierhuizen *et al.*, 1988). Consequently, the increased L-PHA binding after neuraminidase digestion suggests that the polylactosamine oligosaccharides of MDCK LAMP-2 are sialylated as previously described (Carlsson *et al.*, 1988; Granger *et al.*, 1990). The lack of binding of LAMP-2 to the sialic acid-specific lectin LPA may be due to the specificity of that lectin for only some sialic acid derivatives. Smaller amounts of 1- and 3-d LAMP-2 were recognized by the fucose-specific lectin UEA-1. The data presented do not allow conclusions to be drawn concerning either differential sialylation or fucosylation of 1- and 3-d LAMP-2.

The role of terminal processing of N-linked oligosaccharides in the differential glycosylation of LAMP-2 in 1- and 3-d MDCK cultures was corroborated by studies of a ricin-resistant MDCK cell line (Meiss *et al.*, 1982). MDCK-RCA^R cells are incapable of incorporating galactose into the lumen of the Golgi such that complex N-linked oligosaccharide structures are truncated with a terminal GlcNAc preventing the addition of polylactosamine chains (Brändli *et al.*, 1988). LAMP-2 isolated from MDCK-RCA^R cells grown for either 1 or 3 d is significantly smaller than LAMP-2 from 3-d MDCK cells and has the same molecular size (86 kDa) from both 1- and 3-d MDCK cells (Figure 4), suggesting that LAMP-2 from 1- and 3-d MDCK-RCA^R cells contains none of the terminal glycosylation motifs found in 1- and 3-d MDCK cells. This was corroborated by the lack of sensitivity of LAMP-2 from MDCK-RCA^R cells to digestion with either neuraminidase or endo- β -galactosidase.

Figure 4. Polarity of LAMP-2 in MDCK and ricin-resistant MDCK-RCA^R cells. MDCK and MDCK-RCA^R cells cultured for 1 or 3 d on polycarbonate filters were pulse labeled for 20 min with ^{35}S -methionine/cysteine and then chased at 37°C from 10 to 80 min after the end of the pulse with anti-LAMP-2 antibody added to the apical (AP) or basolateral (BL) medium at 37°C, and antigen-antibody complexes were precipitated to detect newly synthesized LAMP-2 targeted to the cell surface.

Polarity of LAMP-2 Delivery in 1- and 3-d MDCK and MDCK-RCA^R Cells

Under the conditions of plating, 2×10^6 cells per 24 mm Transwell, both MDCK and MDCK-RCA^R cell monolayers acquire a transepithelial resistance of $\geq 240 \Omega/\text{cm}^2$ after 1 and 3 d of plating indicating that they have formed a "tight" monolayer enabling the use of the domain selective surface immunoprecipitation technique previously used to define the polarity and targeting of LAMP-2 in MDCK cells (Nabi *et al.*, 1991). The generation of polarity by 1- and 3-d cultures of both MDCK and MDCK-RCA^R cells was demonstrated by the polarized delivery of LAMP-2 exclusively basolaterally using surface immunoprecipitation at 37°C with anti-LAMP-2 mAb (Figure 4). After a 20-min pulse label with ³⁵S-methionine/cysteine, anti-LAMP-2 antibody was added to either the apical or basolateral medium from 10 to 80 min after the pulse and antigen-antibody complexes precipitated. Only antibody added to the basolateral medium was capable of detecting newly synthesized LAMP-2 in 1- and 3-d cultures of both MDCK and MDCK-RCA^R cells. The absence of apical detection of LAMP-2 indicates that the altered glycosylation of LAMP-2 in MDCK and MDCK-RCA^R cells plated for 1 or 3 d does not influence the polarity of its delivery from the TGN to the basolateral domain.

Differential Rate of Basolateral Delivery of LAMP-2 in 1- and 3-d MDCK Cells

Although the polyglactosamine glycosylation of LAMP-2 did not affect its polarity, by using a surface targeting protocol (Nabi *et al.*, 1991), 1- and 3-d LAMP-2 exhibited a differential rate of basolateral delivery (Figure 5A). After a 20-min pulse label with ³⁵S-methionine/cysteine, anti-LAMP-2 antibody was added to the basolateral medium for 15 min time periods at 20-min intervals such that the amount of LAMP-2 arriving at the cell surface during those discrete time periods could be quantitated. Interestingly, the rate of basolateral delivery of LAMP-2 was significantly increased in 3-d cultures relative to 1-d MDCK cultures. This is seen clearly by the peak appearance of LAMP-2 in the 20 and 40 time periods (equivalent to a $t_{1/2}$ of surface delivery of 28 min) (Figure 6) for 3-d MDCK cells and in the 40 and 60 time periods ($t_{1/2} = 40$ min) for 1-d MDCK cells. In MDCK-RCA^R cells the rate of surface delivery is identical in both 1- and 3-d cultures and corresponds to the slower rate of surface delivery in 1-d MDCK cells. The differential delivery rate of LAMP-2 in 1- and 3-d MDCK cultures occurred after arrival of the proteins to the Golgi apparatus as the rate of acquisition of endo H resistance, a cis-Golgi event, was equivalent ($t_{1/2} = 25$ min) (Figure 5B). The narrow time differential between the half-times of acquisition of endo H resistance and basolateral delivery (Figure 6) reflects a very rapid transit of LAMP-2 from the cis-Golgi to the basolateral

Figure 5. Rate of basolateral delivery and acquisition of endo H resistance by LAMP-2 in 1- and 3-d MDCK and MDCK-RCA^R cells. (A) After a 20-min ³⁵S-methionine/cysteine pulse label of MDCK and MDCK-RCA^R cells cultured for 1 or 3 d on polycarbonate filters, basolateral delivery of newly synthesized LAMP-2 was determined by the discrete addition of anti-LAMP-2 antibody to the basolateral medium for 15 min periods at either 0, 20, 40, 60, 80, or 100 min (as indicated) after which antigen-antibody complexes were precipitated. (B) 1-d and 3-d MDCK cells grown on polycarbonate filters were pulse labeled for 20 min with ³⁵S-methionine/cysteine and then chased at 37°C for 0, 10, 20, 30, 40, or 50 min as indicated. LAMP-2 was immunoprecipitated and digested with endoglycosidase H.

domain in 3-d MDCK cells. This rapid rate of delivery correlates with the decreased polyglactosamine glycosylation of 3-d MDCK LAMP-2 suggesting a relationship between rate of passage through the Golgi apparatus and the polyglactosamine glycosylation of LAMP-2.

DISCUSSION

The kidney-derived epithelial cell line, MDCK, is polarized in culture and has been extensively used to study the mechanisms and pathways underlying protein sorting in polarized epithelial cells and signals that target proteins to the apical and basolateral cell surfaces have been identified (Rodriguez-Boulant and Nelson, 1989; Mostov *et al.*, 1992). We have previously demonstrated that an endogenous MDCK LAMP-2 is targeted in a polar fashion to the basolateral early endosome/plasma membrane before delivery to the lysosome (Nabi *et al.*, 1991), a route similar to that described for the biosynthetic pathway of LEP100 (avian LAMP-1) in nonpolarized cells (Mathews *et al.*, 1992). We show that MDCK LAMP-2 exhibits increased polyglactosamine glycosylation in MDCK cells plated for only 1 d. A previous report using lectin precipitation was unable to detect any glycosylation motifs that correlated with apical or

Figure 6. Schematic representation of the basolateral targeting of MDCK LAMP-2. The basolateral targeting and endo H digestion experiments of 1- and 3-d MDCK (top) and MDCK-RCA^R cells (bottom) were quantified by scanning densitometry. The basolateral targeting of 1-d cells is represented by (●) and solid lines and of 3-d cells by (○) and dashed lines. The acquisition of endo H resistance for LAMP-2 in MDCK cells is represented by open symbols and a single dotted line. The data presented is the average of multiple experiments (MDCK, 3; MDCK-RCA^R, 2; endo H, 2), all of which gave similar results, and is presented as the fraction of the maximal value. The time points for the endo H digestion correspond to the exact time at which the cells were transferred to 4°C. Those for the basolateral surface targeting experiments correspond to the end-point of the time period during which the antibody was added. Addition of anti-LAMP-2 antibody at 37°C means that its interaction with basolateral LAMP-2 is cumulative over the 15 min time period. As a result, the best representation of the time of delivery of surface immunoprecipitated LAMP-2 is the end-point of each time period. The addition of antibody for 15 min at the times indicated in Figure 5A (0, 20, 40, 60, 80, 100 minutes) is therefore plotted in Figure 6 at those times plus 15 min (15, 35, 55, 75, 95, 115 min).

basolateral polarity in MDCK cells (Lisanti *et al.*, 1989). However, inhibition of N-glycosylation by tunicamycin results in the nonpolar secretion of a normally apical 80-kDa sulfated glycoprotein (Urban *et al.*, 1987). Recent studies of an apical MDCK sialoglycoprotein, gp114, in MDCK-RCA^R cells suggest that terminal glycosylation might influence polarity as a pool of gp114 was found basolateral in the ricin-resistant cells (Le Bivic *et al.*, 1993). To determine whether increased polylactosamine glycosylation of 1-d MDCK LAMP-2 influences its basolateral polarity, we studied LAMP-2 delivery by surface immunoprecipitation in MDCK and MDCK-RCA^R cells plated for either 1 or 3 d. In both MDCK and

MDCK-RCA^R cells, LAMP-2 is targeted in a polarized fashion to the basolateral domain indicating that polylactosamine glycosylation of LAMP-2 does not affect its polarity and sorting in the TGN. The lack of an effect on the polarity of LAMP-2 or of an apical glycosol phosphatidyl inositol-anchored protein (Lisanti *et al.*, 1990) in MDCK-RCA^R cells suggests that the influence of glycosylation on polarity is protein specific.

Although LAMP-2 polarity was not altered in 1- and 3-d MDCK cells, its rate of basolateral delivery is significantly increased in MDCK cells plated for 3 d relative to MDCK cells plated for only 1 d. The rate differential occurred after arrival of LAMP-2 to the Golgi as the rate of acquisition of endo H resistance, a cis-Golgi event, occurred with equal rates at either day. The narrow differential between acquisition of endo H resistance ($t_{1/2}$ = 25 min) and basolateral delivery ($t_{1/2}$ = 28 min) of LAMP-2 in 3-d cells is indicative of a very rapid transport of the protein through the Golgi apparatus to the basolateral domain. It is not clear whether LAMPs are targeted from the TGN to the plasma membrane or early endosome and whether their TGN-surface route is the same as other plasma membrane proteins (Nabi *et al.*, 1991; Harter and Mellman, 1992; Mathews *et al.*, 1992). Nevertheless, the LAMP-2 delivery rates are comparable to those described for other basolateral membrane proteins (Le Bivic *et al.*, 1990 and others). Considering the equivalent rate of acquisition of endo H resistance for LAMP-2 at both days of plating, the time of transit of LAMP-2 from the cis-Golgi to the basolateral domain is significantly longer (4-fold) in 1-d MDCK cells compared with 3-d MDCK cells. This is likely due to a slower passage of the protein through the Golgi apparatus or to a delay in its exit from the TGN resulting in a significantly extended residence time of newly synthesized LAMP-2 in the Golgi apparatus of 1-d MDCK cells.

The slower rate of basolateral delivery of LAMP-2 in 1-d cells corresponds to the increased presence of polylactosamine glycosylation. The fact that the rate of basolateral delivery of LAMP-2 is the same in MDCK-RCA^R cells plated for either 1 or 3 d underlines the relationship between polylactosamine glycosylation and the differential rate of delivery of 1- and 3-d LAMP-2 in normal MDCK cells. It further argues that increased polylactosamine glycosylation and consequently increased number of glycosyltransferase reactions are not responsible for the slow delivery of LAMP-2 in 1-d MDCK cells. Experiments performed in HL-60 cells, which exhibit decreased polylactosamine glycosylation after differentiation to leukocytes (Lee *et al.*, 1990), have suggested that increased polylactosamine glycosylation is a consequence of a slower passage through the Golgi apparatus. Highly elongated polylactosamine chains were found on LAMP-1 and LAMP-2 isolated from HL-60 cells incubated for extended periods at 20°C, a temperature block that prevents exit from the TGN (Wang

et al., 1991). An extended residence time of LAMP-2 in the Golgi apparatus of 1-d MDCK cells would enable increased interaction with polylectosamine-specific glycosyltransferases resulting in the generation of elongated polylectosamine chains. The results presented here demonstrate that the Golgi transit time of LAMPs might serve to regulate the extent of their polylectosamine glycosylation not only by treatment of cells *in vitro* but also *in vivo*.

The Golgi apparatus is the site of posttranslational modification of N-linked and O-linked glycosylation, and TGN is the site of protein sorting to distinct surface domains in polarized cells (Mellman and Simons, 1992). Transport of proteins through the Golgi is thought to be unidirectional or via "bulk flow" (Pfeffer and Rothman, 1991) yet mechanisms must exist to regulate the specific interactions of proteins with glycosyltransferases to generate protein-specific glycosylation. Cell shape modulation can influence glycosylation as B16-F1 melanoma cells grown in suspension culture exhibit increased expression of sialylated peanut agglutinin-binding O-linked oligosaccharides (Nabi and Raz, 1987). In MDCK cells the organization of the Golgi apparatus changes with time in culture from compact and localized near the microtubule organizing center at 1 d, to spread around the nucleus at 3 d, and finally, at 5 d, to the apiconuclear Golgi localization characteristic of well-polarized epithelia (Bacallao *et al.*, 1989). These morphological studies were performed using cells plated at a lower density (2×10^4 cells/cm²) than those described here (4×10^5 cells/cm²) such that the timing of the described changes in Golgi structure may not correspond directly with the changes in the rate of basolateral delivery between 1- and 3-d cells that we have observed. It is conceivable that the increased density of plating, at confluency, serves to speed up the changes in Golgi morphology such that in our hands a spread Golgi morphology is achieved after only 1 d and an apiconuclear Golgi localization after 3 d. If so, dispersal of the Golgi may be responsible for the slow delivery at 1 d and the formation of a compact, organized Golgi apparatus responsible for the efficient transit of LAMP-2 through the Golgi apparatus at 3 d.

The slow rate of delivery of LAMP-2 in both 1- and 3-d MDCK-RCA^R cells could be due to the fact that the cells were isolated from a mutated cell population and, while exhibiting functional polarity, do not acquire the organized epithelial phenotype of normal MDCK cells. In CHO cells, disruption of Golgi architecture by colcemid did not alter the structure of N-linked glycopeptides nor the bulk rate of delivery of glycoproteins to the cell surface (Stults *et al.*, 1989b). Extension of polylectosamine chains requires the repeated sequential addition of only two oligosaccharides such that this glycosylation motif might be particularly sensitive to residence time in the Golgi apparatus. Such an influence of cellular and Golgi organization on polylectosamine

glycosylation could explain the differential degree of LAMP polylectosamine glycosylation found during cellular differentiation and transformation (Fukuda, 1985; Heffernan *et al.*, 1989; Youakim *et al.*, 1989; Amos and Lotan, 1990; Lee *et al.*, 1990; Saitoh *et al.*, 1992).

Regulation of differential LAMP polylectosamine glycosylation during cellular differentiation is likely more complex than just the rate of biosynthetic transit through the Golgi apparatus. Polylectosamines are preferentially added to a β 1,6-mannose linkage in tri and tetra antennary N-linked structures (Cummings and Kornfeld, 1984; Dennis *et al.*, 1987; Sasaki *et al.*, 1987; Van den Eijnden *et al.*, 1988) reflecting interactions with specific glycosyltransferases responsible for β 1,6 branching and for chain extension by the addition of GlcNAc to a terminal galactose residue (Schacter, 1986; Van den Eijnden *et al.*, 1988). Increased expression of β -1,6-N-acetylglucosaminyltransferase activity is associated with the increased polylectosamine glycosylation of LAMP-1 in metastatic tumor cell lines (Yousefi *et al.*, 1991). Expression of specific glycosyltransferases must therefore be implicated in polylectosamine glycosylation and may be responsible for the increased polylectosamine glycosylation of LAMP-2 in 1-d MDCK cells. Furthermore, we cannot exclude the possibility that differential glycosyltransferase expression in 3-d MDCK cells results in the expression of a distinct oligosaccharide motif on LAMP-2 responsible for its rapid transit through and/or exit from the Golgi that would not be expressed on the truncated oligosaccharides of MDCK-RCA^R cells. No evidence for such an oligosaccharide structure was detected by the glycosidase digestions and lectin binding studies performed here and further characterization of the oligosaccharide structure of LAMP-2 from 1- and 3-d MDCK cells would be required.

The data presented suggest an intricate relationship between cellular organization, the rate of Golgi transit, and the polylectosamine glycosylation of an endogenous MDCK LAMP-2. The increased polylectosamine expression of LAMPs at early times after plating in both the intestine derived CaCo-2 (Youakim *et al.*, 1989) and kidney-derived MDCK epithelial cell lines suggests a functional role for the polylectosamine glycosylation of LAMPs in early events during establishment of a polarized epithelial monolayer.

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REFERENCES

- Amos, B., and Lotan, R. (1990). Modulation of lysosomal-associated membrane glycoproteins during retinoic acid-induced embryonal carcinoma cell differentiation. *J. Biol. Chem.* 265, 19192–19198.
- Bacallao, R., Antony, C., Dotti, C., Karsenti, E., Stelzer, E., and Simons, K. (1989). The subcellular organization of Madin-Darby canine kidney cells during the formation of a polarized epithelium. *J. Cell Biol.* 109, 2817–2832.
- Barriocanal, J.G., Bonifacio, J.S., Yuan, L., and Sandoval, I.V. (1986). Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem.* 261, 16755–16763.
- Bierhuizen, M.F.A., Edzes, H.T., Schiphorst, W.E.C.M., van den Eijnden, D.H., and van Dijk, W. (1988). Effect of $\alpha(2-6)$ -linked sialic acid and $\alpha(1-3)$ -linked fucose on the interaction of N-linked glycopeptides and related oligosaccharides with immobilized *Phaseolus vulgaris* leucoagglutinating lectin (L-PHA). *Glycoconjugate J.* 5, 85–97.
- Brändli, A.W., Hansson, G.C., Rodriguez-Boulán, E., and Simons, K. (1988). A polarized epithelial cell mutant deficient in translocation of UDP-galactose into the Golgi complex. *J. Biol. Chem.* 263, 16283–16290.
- Carlsson, S.R., and Fukuda, M. (1990). The polylactosamines of human lysosomal membrane glycoproteins lamp-1 and lamp-2. *J. Biol. Chem.* 265, 20488–20495.
- Carlsson, S.R., Roth, J., Piller, F., and Fukuda, M. (1988). Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* 263, 18911–18919.
- Cha, Y., Holland, S.M., and August, J.T. (1990). The cDNA sequence of mouse LAMP-2. *J. Biol. Chem.* 265, 5008–5013.
- Cooper, D.N.W., Massa, S.M., and Barondes, S.H. (1991). Endogenous muscle lectin inhibits myoblast adhesion to laminin. *J. Cell Biol.* 115, 1437–1448.
- Cummings, R.D., and Kornfeld, S. (1982). Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leucoagglutinating and erythroagglutinating lectins. *J. Biol. Chem.* 257, 11230–11234.
- Cummings, R., and Kornfeld, S. (1984). The distribution of repeating [Gal β 1,4GlcNAc β 1,3] sequences in asparagine-linked oligosaccharides of the mouse lymphoma cell lines BW5147 and PHA^R2.1. *J. Biol. Chem.* 259, 6253–6260.
- Dennis, J., Laferté, S., Waghorne, C., Breitman, M., and Kerbel, R. (1987). β 1,6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 236, 582–585.
- Do, K.-Y., Smith, D.F., and Cummings, R.D. (1990). LAMP-1 in CHO cells is a primary carrier of poly-N-acetylactosamine chains and is bound preferentially by a mammalian S-type lectin. *Biochem. Biophys. Res. Comm.* 173, 1123–1128.
- Febbraio, M., and Silverstein, R.L. (1990). Identification and characterization of LAMP-1 as an activation-dependent platelet surface glycoprotein. *J. Biol. Chem.* 265, 18531–18537.
- Feizi, T. (1991). Carbohydrate differentiation antigens: probably ligands for cell adhesion molecules. *Trends Biochem. Sci.* 16, 84–86.
- Fukuda, M. (1985). Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta* 780, 119–150.
- Fukuda, M. (1991). Lysosomal membrane glycoproteins. *J. Biol. Chem.* 266, 21327–21330.
- Fukuda, M., Viitala, J., Matteson, J., and Carlsson, S.R. (1988). Cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* 263, 18920–18928.
- Fukuda, M., Watanabe, K., and Hakamori, S. (1978). Release of oligosaccharides from various sphingolipids by endo- β -galactosidase. *J. Biol. Chem.* 253, 6814–6819.
- Granger, B.L., Green, S.A., Gabel, C.A., Howe, C.L., Mellman, I., and Helenius, A. (1990). Characterization and cloning of lgp110, a lysosomal membrane glycoprotein from mouse and rat cells. *J. Biol. Chem.* 265, 12036–12043.
- Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988). The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52, 329–341.
- Harter, C., and Mellman, I. (1992). Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol.* 117, 311–325.
- Heffernan, M., Yousefi, S., and Dennis, J.W. (1989). Molecular characterization of P2B/LAMP-1, a major protein target of a metastasis-associated oligosaccharide structure. *Cancer Res.* 49, 6077–6084.
- Kaplan, H., Welply, J., and Lennarz, W. (1987). Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly. *Biochim. Biophys. Acta* 906, 161–173.
- Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5, 483–525.
- Le Bivic, A., Garcia, M., and Rodriguez-Boulán, E. (1993). Ricin resistant MDCK cells mis-sort a major endogenous apical sialoglycoprotein. *J. Biol. Chem.* 268, 6909–6916.
- Le Bivic, A., Sambuy, Y., Mostov, K., and Rodriguez-Boulán, E. (1990). Vectorial targeting of an endogenous apical membrane sialoglycoprotein and uvomorulin in MDCK cells. *J. Cell Biol.* 110, 1533–1539.
- Lee, N., Wang, W.-C., and Fukuda, M. (1990). Granulocytic differentiation of HL-60 cells is associated with increase of poly-N-acetylactosamine in Asn-linked oligosaccharides attached to human lysosomal membrane glycoproteins. *J. Biol. Chem.* 265, 20476–20487.
- Leffler, H., and Barondes, S.H. (1986). Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian β -galactosides. *J. Biol. Chem.* 261, 10119–10126.
- Lippincott-Schwartz, J., and Fambrough, D.M. (1986). Lysosomal membrane dynamics. Structure and interorganelle movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* 102, 1593–1605.
- Lippincott-Schwartz, J., and Fambrough, D.M. (1987). Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. *Cell* 49, 669–677.
- Lis, H., Joubert, F.J., and Sharon, N. (1985). Isolation and properties of N-acetylactosamine-specific lectins from nine *Erythrina* species. *Phytochemistry* 24, 2803–2809.
- Lisanti, M.P., Le Bivic, A., Saltiel, A.R., and Rodriguez-Boulán, E. (1990). Preferred apical distribution of GPI-anchored proteins: a highly conserved feature of the polarized epithelial phenotype. *J. Membr. Biol.* 113, 155–167.
- Lisanti, M.P., Le Bivic, A., Sargiacomo, M., and Rodriguez-Boulán, E. (1989). Steady-state distribution and biogenesis of endogenous Madin-Darby canine kidney glycoproteins: evidence for intracellular sorting and polarized cell surface delivery. *J. Cell Biol.* 109, 2117–2127.
- Lotan, R., Lotan, D., and Carretero, D. (1989). Modulation of galactoside-binding lectins in tumor cells by differentiation-inducing agents. *Cancer Lett.* 48, 115–122.
- Mane, S., Marzella, L., Bainton, D., Holt, V., Cha, Y., Hildreth, J., and August, J. (1989). Purification and characterization of human lysosomal membrane glycoproteins. *Arch. Biochem. Biophys.* 268, 360–378.
- Mathews, P., Martinie, J., and Fambrough, D. (1992). The pathway and targeting signal for delivery of the integral membrane glycoprotein LEP100 to lysosomes. *J. Cell Biol.* 118, 1027–1040.

- Meiss, H.K., Green, R.F., and Rodriguez-Boulon, E. (1982). Lectin-resistant mutants of polarized epithelial cells. *Mol. Cell. Biol.* 2, 1287-1294.
- Mellman, I., and Simons, K. (1992). The Golgi complex: in vitro veritas. *Cell* 68, 829-840.
- Merkle, R.K., and Cummings, R.D. (1987). Relationship of the terminal sequences to the length of poly-N-acetylglucosamine chains in asparagine-linked oligosaccharides from the mouse lymphoma cell line BW5147. *J. Biol. Chem.* 262, 8179-8189.
- Merkle, R.K., and Cummings, R.D. (1988). Asparagine-linked oligosaccharides containing poly-N-acetylglucosamine chains are preferentially bound by immobilized calf heart agglutinin. *J. Biol. Chem.* 263, 16143-16149.
- Mostov, K., Apodaca, G., Aroeti, B., and Okamoto, O. (1992). Plasma membrane sorting in polarized epithelial cells. *J. Cell Biol.* 116, 577-583.
- Nabi, I.R., Le Bivic, A., Fambrough, D., and Rodriguez-Boulon, E. (1991). An endogenous MDCK lysosomal membrane glycoprotein is targeted basolaterally before delivery to lysosomes. *J. Cell Biol.* 115, 1573-1584.
- Nabi, I.R., and Raz, A. (1987). Cell shape modulation alters glycosylation of a metastatic melanoma cell surface antigen. *Int. J. Cancer* 40, 396-401.
- Ochieng, J., Gerold, M., and Raz, A. (1992). Dichotomy of the laminin-binding properties of soluble and membrane-bound human galactoside-binding protein. *Biochem. Biophys. Res. Commun.* 186, 1674-1680.
- Pfeffer, S., and Rothman, J. (1991). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56, 829-852.
- Rodriguez-Boulon, E., and Nelson, W.J. (1989). Morphogenesis of the polarized epithelial phenotype. *Science* 245, 718-725.
- Saitoh, O., Wang, W.-C., Lotan, R., and Fukuda, M. (1992). Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. *J. Biol. Chem.* 267, 5700-5711.
- Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987). Carbohydrate structure of erythropoietin in Chinese hamster ovary cells by a human erythropoietin cDNA. *J. Biol. Chem.* 262, 12059-12076.
- Sato, S., and Hughes, R.C. (1992). Binding specificity of a baby hamster kidney lectin for H type I and II chains, polyglucosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. *J. Biol. Chem.* 267, 6983-6990.
- Schacter, H. (1986). Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem. Cell Biol.* 64, 164-181.
- Stults, N.L., Asta, L.M., and Lee, Y.C. (1989a). Immobilization of proteins on oxidized crosslinked sepharose preparations by reductive amination. *Anal. Biochem.* 180, 114-119.
- Stults, N.L., Fechner, M., and Cummings, R.D. (1989b). Relationship between Golgi architecture and glycoprotein biosynthesis and transport in Chinese hamster ovary cells. *J. Biol. Chem.* 264, 19956-19966.
- Urban, J., Parczyk, K., Leutz, A., Kaye, M., and Kondor-Koch, C. (1987). Constitutive apical secretion of an 80-kD sulfated glycoprotein complex in the polarized epithelial Madin-Darby canine kidney cell line. *J. Cell Biol.* 105, 2735-2743.
- Van den Eijnden, D., Koenderman, A., and Schiphorst, W. (1988). Biosynthesis of blood group i-active polyglucosaminoglycans. *J. Biol. Chem.* 263, 12461-12471.
- Wang, W.-C., Lee, N., Aoki, D., Fukuda, M., and Fukuda, M. (1991). The poly-N-acetylglucosamines attached to lysosomal membrane glycoproteins are increased by prolonged association with the Golgi complex. *J. Biol. Chem.* 266, 23185-23190.
- Woo, H.J., Shaw, L.M., Messier, J.M., and Mercurio, A.M. (1990). The major nonintegrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2). *J. Biol. Chem.* 265, 7097-7099.
- Youakim, A., Romero, P.A., Yee, K., Carlsson, S.R., Fukuda, M., and Herscovics, A. (1989). Decrease in polyglucosamines associated with lysosomal membrane glycoproteins during differentiation of CaCo-2 human colonic adenocarcinoma cells. *Cancer Res.* 49, 6889-6895.
- Yousefi, S., Higgins, E., Daoling, Z., Pollex-Kruger, A., Hindsgaul, O., and Dennis, J.W. (1991). Increased UDP-GlcNAc:Gal β 1-3GalNAc-R (GlcNAc to GalNAc) β 1-6-N-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. *J. Biol. Chem.* 266, 1772-1782.
- Zhou, Q., and Cummings, R.D. (1990). The S-type lectin from calf heart tissue binds selectively to the carbohydrate chains of laminin. *Arch. Biochem. Biophys.* 281, 27-35.