Sorting protein-related receptor (SorLA/LR11) is a highly conserved mosaic receptor that is expressed by cells in a number of different tissues including principal cells of the collecting ducts in the kidney and neurons in the central and peripheral nervous systems. SorLA/LR11 has features that indicate it serves as a sorting receptor shuttling between the plasma membrane, endosomes, and the Golgi. We have found that a fraction of SorLA/LR11 that is synthesized in the kidney and the brain bears N-linked oligosaccharides that are modified with terminal β1,4-linked GalNAc-4-SO4. Oligosaccharides located in the vacuolar sorting (Vps) 10p domain (Vps10p domain) are modified with β1,4-linked GalNAc when the Vps10p domain is expressed in cells along with either of two recently cloned protein-specific β1,4GalNAc-transferases, GalNAcTIII and GalNAcTIV. Either of two sequences with basic amino acids located within the Vps10p domain is able to mediate recognition by these β1,4GalNAc-transferases. The highly specific modification of oligosaccharides in the Vps10p domain of SorLA/LR11 with terminal GalNAc-4-SO4 suggests that this unusual modification may modulate the interaction of SorLA/LR11 with proteins and influence their trafficking.

N-Linked Oligosaccharides on the Low Density Lipoprotein Receptor Homolog SorLA/LR11 Are Modified with Terminal GalNAc-4-SO4 in Kidney and Brain*

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The abbreviations used are: SorLA, sorting protein-related receptor; LDLR, low density lipoprotein receptor; TPER, tissue protein extraction reagent; PVDF, polyvinylidene difluoride; UB, unbound; CHO, Chinese hamster ovary; HNK, human embryonic kidney; HPLC, high pressure liquid chromatography; WFA, Wisteria floribunda agglutinin; HRP, horseradish peroxidase.

The receptor migrates with Mr of over 300,000 when examined by SDS-PAGE indicating that a large fraction of the 20 potential Asn-glycosylation sites present on the luminal domain are utilized. We have determined that a fraction of SorLA/LR11 produced in the kidney and brain bears N-linked oligosaccharides that terminate with β1,4-linked GalNAc-4-SO4. We first described N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-SO4 on the glycoprotein hormone lutropin (8). A limited number of other glycoproteins including thyrotropin (8–11), proopiomelanocortin (12–14), tenascin-R (15, 16), carbonic anhydrase VI (17), Tamm-Horsfall glycoprotein (18, 19), and sialoadhesin (20) also bear these novel sulfated oligosaccharides. Terminal GalNAc-4-SO4 on N-linked oligosaccharides is recognized by the Mannose/GalNAc-4-SO4 receptor, expressed by hepatic endothelial cells of mammals, which mediates the clearance of glycoproteins bearing these structures from the blood (21–24). The cysteine-rich domain, a region located near the amino terminus of the mannose/GalNAc-4-SO4 receptor, accounts for GalNAc-4-SO4 binding (25). We have used a chimeric protein termed Cys-Fc, which consists of the cysteine-rich domain and the constant region of human IgG1, to identify tissues and specific cells that express glycoproteins bearing terminal GalNAc-4-SO4 and to isolate these glycoproteins by affinity chromatography (15, 16, 25). In this report we provide evidence that SorLA/LR11 is among the small family of glycoproteins that are modified with N-linked oligosaccharides that terminate with β1,4-linked GalNAc-4-SO4.
EXPERIMENTAL PROCEDURES

**Histochemical Staining**—Mouse kidneys were removed and frozen with Tissue-Tek O.C.T. (Sakura Finetek U.S.A., Inc., Torrance, CA) on dry ice. Ten-μm cryosections were fixed with Methacarn solution (methanol:chloroform:acetic acid, 7:2:1) for 10 min at 20 °C. The sections were rehydrated in decreasing ethanol concentrations. After washing in phosphate-buffered saline, endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol for 30 min followed by washing with phosphate-buffered saline. Sections were incubated in 5% goat serum in TNB (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% casein) for 30 min at 25 °C to inhibit nonspecific binding. After blocking sections were incubated with Cys-Fc (10 ng/ml in TNB) overnight at 4 °C. Sections were washed 3 times with TNT (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then incubated with peroxidase-conjugated goat anti-human IgG, Fc-γ fragment specific (Jackson ImmunoResearch Laboratories, Inc.). Slides were washed again 3 times with TNT and stained with Sigma Fast 3,3-diaminobenzidine (Sigma). Slides were counterstained with Harris Hematoxylin Solution (Sigma). After dehydrating and clearing, the slides were mounted with DPX (Fluka Biochemika, Ronkonkoma, NY).

**Cys-Fc-Agarose Preparation**—Versalinx technology (Calbiochem) was used to covalently attach Cys-Fc to agarose. Versalinx amine modifying reagent was incubated with Cys-Fc at a molar ratio of 10:1. The phenyldiboronic acid-modified Cys-Fc was then incubated with Versalinx SHA-agarose. The conjugate contained 10–20 mg of Cys-Fc per ml of agarose.

**Western Blot Analyses**—Samples were analyzed by SDS-PAGE using 3–8% NuPAGE Tris acetate polyacrylamide gels (Invitrogen). Molecular weight standards, Precision Plus Protein Dual Color Standards (Bio-Rad), were included to determine the apparent mobilities of proteins of interest. Following electrophoretic transfer to PVDF membranes (Millipore, Billerica, MA) the membranes were blocked with 1% casein (Hammersten grade, BDH Chemicals) and then analyzed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) for detection of HRP-labeled probes. Cys-Fc-HRP was prepared by conjugating HRP to Cys-Fc (25) using the EZ-link Activated Peroxidase Antibody Labeling Kit (Pierce). Incubations with Cys-Fc-HRP were carried out between 24 and 72 h at 4 °C. Rabbit antibody directed against the fibronectin domain of SorLA/LR11 (26) was provided by Dr. Chica Shaller (Universitat Hamburg, Hamburg, Germany). Constructs containing the Vps10p domain of SorLA/LR11 with a V5 epitope at the carboxyl terminus were detected using HRP-anti-V5 (Invitrogen). Mouse anti-Tenascin-R/Janusin (catalog number 610629) was purchased from BD Biosciences.

**Purification of Cys-Fc Reactive Glycoproteins from Kidney**—The medullas from 50 mouse kidneys were excised, frozen in liquid nitrogen, and fractured into a powder using a Bio-Pulservizer (BioSpec Products, Bartlesville, OK). The powder was suspended in ice-cold tissue protein extraction reagent (TPER) (Pierce Chemicals) containing EDTA-free Complete protease inhibitor (Roche, Nutley, NJ), 5 ml/g of powder, using a Polytron (Brinkmann). The extract was centrifuged at 10,000 × g for 5 min and the supernatant collected. The supernatant was incubated with the anion exchanger Vivapure-D (Vivasciences, Carlsbad, CA). Bound proteins were eluted with a step gradient consisting of 0.1, 0.5, 1.0, 1.5, and 2.0 mM NaCl in TPER. Cys-Fc reactive proteins were eluted in the 0.5 and 1.0 mM NaCl fractions as determined by Western blot analysis following separation on NuPAGE gels. The fractions containing Cys-Fc reactive protein were pooled, dialyzed against TPER, and then incubated with 150 μl of Cys-Fc-agarose at 4 °C for 48 h. The Cys-Fc-agarose was collected by brief sedimentation and washed 4 times with 3000 μl of ice-cold TPER for 30 min. The bound proteins were eluted by incubation 3 times with 300 μl of ice-cold TPER con-
Containing 1 mM GalNAc-4-SO₄ for 30 min. The fractions were analyzed by Western blot on 3–8% Tris acetate NuPAGE gels as shown in Fig. 2.

**MS/MS Peptide Sequence Analysis**—A protein corresponding to the Cys-Fc reactive material seen by Western blot was visualized with Colloidal Coomassie Blue G-250 and excised from the acrylamide gel for sequence analysis. Proteolytic digestion with trypsin, separation of peptides by microparticulate reverse-phase HPLC, nano-electrospray tandem mass spectrometry on a Finnigan LCQ DECA XP quadrupole ion trap mass spectrometer, and correlation of the fragments with known sequences using the Sequest algorithm were performed by the Harvard Microchemistry Facility, Harvard, University, Cambridge, MA. A total of 33 distinct peptides were identified that corresponded to the major component mouse SorLA/LR11 were identified.

**Analytic Separation of Cys-Fc Reactive Glycoproteins from Kidney**—Following identification of the major Cys-Fc reactive band as SorLA/LR11 analytic separations were carried out. Medullas from 10 mouse kidneys were excised, minced, placed in 300 μl of ice-cold TPER containing EDTA-free Complete protease inhibitor, and ground using a PlusOne Sample Grinding Kit (Amersham Biosciences). The extract was diluted with an additional 700 μl of TPER containing EDTA-free Complete protease inhibitor and incubated for 1 h on ice with mixing. The sample was sedimented a 10,000 × g for 10 min. The supernatant was incubated with 50 μl of Cys-Fc-agarose in a Mini-Spin column (product number 69705, Pierce) at 4 °C for 5 days. The unbound fraction was then washed 2 times with 100 μl of ice-cold TPER followed by elution by incubation with cold TPER containing 1 mM GalNAc-4-SO₄ for 2 h followed by collection by sedimentation at 50 × g. The column was then washed 2 times with 100 μl of cold TPER containing cold TPER followed by collection by sedimentation at 50 × g. This was repeated a total of 4 times. Each of the fractions from the Cys-Fc-agarose column was analyzed by Western blotting using Cys-Fc and anti-SorLA/LR11 as shown in Fig. 3.

**Expression of SorLA/LR11 Constructs**—Human SorLA/LR11 cDNA in the expression plasmid pcDNA3.1/Zeo+ (6) was kindly provided by Dr. Claus M. Petersen, Department of Medical Biochemistry, University of Aarhus, Denmark. The amino-terminal region containing the Vps10p domain was amplified and ligated into pcDNA3.1VsorLA/Vps10p (V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'. Mutations were introduced into the Vps10p domain of pcDNA3.1hSorLA/Vps10p(V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'. Mutations were introduced into the Vps10p domain of pcDNA3.1hSorLA/Vps10p(V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'. Mutations were introduced into the Vps10p domain of pcDNA3.1hSorLA/Vps10p(V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'. Mutations were introduced into the Vps10p domain of pcDNA3.1hSorLA/Vps10p(V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'. Mutations were introduced into the Vps10p domain of pcDNA3.1hSorLA/Vps10p(V5His) by PCR-mediated overlap extension using the following primers: 1) deletion of KPLRRKR, 5'-CGCGCGCGCGAGAGCAGCCGT-GCCCTGCAACGCGAGCCCATC-3' and 5'-GATCT-TTCCGATAGCCTCTC-3'.

**TABLE 1**

| Peptide sequence | Amino acids | Peptide sequence | Amino acids |
|------------------|-------------|------------------|-------------|
| SQVEILASQLGLMGDK | 992–1008    | ADLDDLHSG       | 206–214     |
| YDLAGSQTDEQLPSGLR | 772–788    | VLPIYQPSDDYVVK  | 1912–1927   |
| SVILSDLPHPYIAVFKP | 954–970    | ITFSOQQR        | 946–953     |
| WESFYDPSQDLFYIAVVK | 1953–1971 | YSTNBDETWK      | 563–572     |
| NLLINALYTVR       | 1712–1722  | NLQLSLNR        | 1664–1666   |
| TOGDPSLAPEHILTR   | 1825–1840  | SYNDGAAYR       | 905–914     |
| ENQEVILEEVR        | 288–298    | EYIVEYSR        | 1685–1692   |
| TNVTISSAGAR        | 518–529    | VLRPDRTTVKQ     | 1616–1626   |
| DEQYLFLVR          | 1903–1911  | TFAVNPQDQFR     | 856–866     |
| STVFITIFPSNK       | 594–604    | KIEVANPDQDFR    | 855–866     |
| SYMDSGAAVR         | 905–914    | GSSPPAPL5K      | 1841–1850   |
| ITTVSLAPDLK        | 2020–2032  | VVGEISWQK       | 1594–1601   |
| VHNNDPFPVLR        | 1635–1645  | SVVTFQDK        | 446–466     |
| TPEGIFLOAPR        | 1646–1655  | LVSEDVK         | 915–921     |
| HEPFGSTVLR         | 269–279    | SSDVVSYDYGK     | 132–143     |
| YPANEFHADFRH       | 405–416    | AAVALDFDYER     | 789–799     |
| TVTVSKEQYQLFLVR    | 1897–1911  |                  |             |
CATGCCACATGC-3' and 5'-GCATGTGGCATGGGGG-TCT-3'; and 3) deletion of RRTGRYK, 5'-AAGACTGTGGTTCACCCCACTGCACTGTTCAATTG-3' and 5'-GTTGAAACAGTCTTGTGTCCAGCAACCACCTC-3'. Multiple mutations were introduced sequentially. Expression plasmids were introduced into cultured cells by transfection using Lipofectamine PLUS Reagent or Lipofectamine 2000 (Invitrogen) using protocols recommended by the manufacturer.

RESULTS

Cys-Fc Reactive Material Is Located in the Medulla and Papilla of the Mouse Kidney—We previously reported that both protein-specific β1,4GalNAc-transferase activity and GalNAc-4-sulfotransferase activity can be detected in a number of tissues including the pituitary, salivary glands, brain, and kidney (27). Because the Cys-rich domain of the Man/GalNAc-4-SO₄ receptor is highly specific for terminal sulfated sugars such as β1,4-linked GalNAc-4-SO₄ (25, 28, 29), we utilized a chimeric protein consisting of the Cys-rich domain and the Fc domain of human IgG, Cys-Fc, to immunostain tissue sections for the presence of glycoproteins bearing terminal β1,4-linked GalNAc-4-SO₄ (15, 25). Cells comprising collecting ducts in the medulla and papilla of the mouse kidney are intensely stained (Fig. 1). The immunoreactivity seen in the kidney in combination with the presence of protein-specific β1,4GalNAc-transferase activity and GalNAc-4-sulfotransferase activity indicate that one or more of the glycoproteins produced by these cells are potentially modified with terminal β1,4-linked GalNAc-4-SO₄ on their N- or O-linked oligosaccharides.

Proteins were solubilized from the cortex and medulla of mouse kidneys using TPER and examined by Western blotting following separation by SDS-PAGE and electrophoretic transfer to PVDF membranes. A glycoprotein with $M_r > 250,000$ is the predominant species that reacts with the Cys-Fc chimera and is enriched in the extract of the medulla as compared with the cortex (not shown). This protein was bound by the anion exchanger Vivapure D and eluted at a NaCl concentration between 0.5 and 1.0 M. When the material eluted from the anion exchanger was incubated with Cys-Fc-agarose, the Cys-Fc reactive band was removed from the extract (Fig. 2, lane T versus UB). Consistent with the modest affinity we have observed for glycoproteins with a limited number of GalNAc-4-SO₄ termini (28), the Cys-Fc reactive material was present in the fractions eluted with 150 mM NaCl (Fig. 2, W1–W4) and in the fractions eluted with 1 mM GalNAc-4-SO₄ (Fig. 2, E1–E3). Staining with Coomassie Blue revealed the presence of a protein band at the position of the Cys-Fc reactive material eluted with 1 mM GalNAc-4-SO₄. The fractions eluted with NaCl contained a large number of additional bands when stained with Coomassie Blue (not shown). We therefore utilized the GalNAc-4-SO₄ eluted material for MS/MS analysis.

MS/MS analysis of the protein eluted with GalNAc-4-SO₄ following proteolytic digestion with trypsin and separation of the resulting peptides by microcapillary HPLC yielded 33 peptides that corresponded to the glycoprotein SorLA/LR11 when analyzed by Sequest (Table 1). A non-erythroid form of spectrin was also identified in this sample, however, because spectrin is not glycosylated this was not pursued further. SorLA/LR11 is a member of the LDL-receptor family that is expressed in collecting ducts of the medulla and papilla of murine kidney (31).

SorLA/LR11 Produced in Kidney and Brain Is Bound by Immobilized Cys-Fc—An extract of mouse kidney was prepared using TPER and Cys-Fc reactive glycoproteins were enriched by affinity chromatography as described above. As seen during the larger scale purification, the major Cys-Fc reactive glycoprotein is present in both the NaCl-eluted wash fractions and the GalNAc-4-

FIGURE 3. The major affinity purified, Cys-Fc reactive glycoprotein from kidney corresponds to SorLA/LR11. An extract of kidney medulla was prepared using TPER and incubated with Cys-Fc-agarose. Aliquots (0.4%) of the extract prior to incubation with Cys-Fc (lane T) and after incubation with Cys-Fc (lane marked UB) and aliquots (20%) of the wash fractions (lanes marked W1 and W2) and GalNAc-4-SO₄ eluted fractions (lanes marked E1–E4) were analyzed by SDS-PAGE and Western blot analysis using Cys-Fc (A) and anti-SorLA/LR11 (B). The location of the major Cys-Fc reactive band is indicated by the arrow. Molecular weight standards are indicated by the arrowheads.

FIGURE 4. SorLA/LR11 expressed in brain is bound by Cys-Fc. An extract of mouse brain was prepared using TPER and affinity purified using Cys-Fc-agarose. After washing with TPER, the bound proteins were eluted with TPER containing 1 mM GalNAc-4-SO₄. Aliquots (0.4%) of the extract (T) and unbound (UB) fractions and aliquots (20%) of the wash (W) and GalNAc-4-SO₄ eluted (E) fractions were examined by Western blot analysis following separation by SDS-PAGE and electrophoretic transfer to PVDF using A, Cys-Fc; B, anti-SorLA/LR11; and C, anti-Tenascin-R. The bands corresponding to SorLA/LR11 (filled arrow) and Tenascin-R (open arrows) are indicated. The most rapid migrating form of Tenascin-R does not contain the amino-terminal region that was used to raise the anti-Tenascin-R and is not detected by this antibody (15, 16).
but not CHO cells are able to add terminal GalNAc-4-SO₄ to tenascin-R is modified with terminal GalNAc-4-SO₄ and can be expressed in the brain (27), and Cys-Fc reactive material is particularly prominent in the cerebellum and hippocampus (15, 16). We recently reported that the extracellular matrix protein tenascin-R is abundantly expressed by neurons in the brain (2, 4, 32, 33). Furthermore, GalNAc-4-sulfotransferase and protein-specific β₁,4GalNAc-transferase activities are also expressed in the brain (27), and Cys-Fc reactive material is particularly prominent in the cerebellum and hippocampus (15, 16). We recently reported that the extracellular matrix protein tenascin-R is modified with terminal GalNAc-4-SO₄ and can be bound to immobilized Cys-Fc (15). As can be seen in Fig. 4, a fraction of SorLA/LR11 (A and B, solid arrow), like tenascin-R (A and C, open arrows), is bound by immobilized Cys-Fc and eluted with GalNAc-4-SO₄. This suggests that a subset of the SorLA/LR11 molecules produced in the kidney is modified with terminal GalNAc-4-SO₄.

SorLA/LR11 is abundantly expressed by neurons in the brain (2, 4, 32, 33). Furthermore, GalNAc-4-sulfotransferase and protein-specific β₁,4GalNAc-transferase activities are also expressed in the brain (27), and Cys-Fc reactive material is particularly prominent in the cerebellum and hippocampus (15, 16). We recently reported that the extracellular matrix protein tenascin-R is modified with terminal GalNAc-4-SO₄ and can be bound to immobilized Cys-Fc (15). As can be seen in Fig. 4, a fraction of SorLA/LR11 (A and B, solid arrow), like tenascin-R (A and C, open arrows), is bound by immobilized Cys-Fc and eluted with GalNAc-4-SO₄. This suggests that a subset of the SorLA/LR11 molecules produced in the kidney is modified with terminal GalNAc-4-SO₄.

**Recombinant SorLA/LR11 Is Modified with Terminal GalNAc-4-SO₄ When Expressed in HEK-293 Cells—HEK-293 cells but not CHO cells are able to add terminal GalNAc-4-SO₄ to Asn-linked oligosaccharides on glycoproteins containing the appropriate peptide recognition determinant, such as the glycoprotein hormone LH (34, 35). A fraction of recombinant human SorLA/LR11 synthesized in HEK-293 cells endogenously expressing β₁,4-GalNAc-transferase and GalNAc-4-sulfotransferase activities required for GalNAc-4-SO₄ addition was bound by immobilized Cys-Fc and eluted with GalNAc-4-SO₄ (Fig. 5, lower panel). In contrast, none of the recombinant SorLA/LR11 synthesized in CHO cells, which do not express the required transferases, was bound by immobilized Cys-Fc (Fig. 5, upper panel). The fraction of recombinant SorLA/LR11 produced in HEK-293 cells that was bound by immobilized WFA, which binds terminal β₁,4-linked GalNAc (36, 37), was higher than the fraction of SorLA/LR11 bound by immobilized Cys-Fc (not shown). This indicates that much of the GalNAc added to the recombinant SorLA/LR11 is not further modified with sulfate. Expression of recombinant GalNAc-4-ST1 (38) along with recombinant SorLA/LR11 in HEK-293 cells reduces the amount of SorLA/LR11 bound by immobilized WFA and increases the amount bound by Cys-Fc (not shown). Thus the level of endogenous GalNAc-4-sulfotransferase is not sufficient.

**FIGURE 5. Recombinant SorLA expressed in HEK-293 cells is bound by immobilized Cys-Fc.** Recombinant mouse SorLA/LR11 expressed in CHO (upper panel) and HEK-293 (lower panel) cells was incubated with 50 μl of Cys-Fc-phenylboronic acid SHA-agarose. Ten μl of the unbound 5-ml fraction (UB) and 3 successive 200-μl washes (W1, W2, and W3) and 20 μl of 3 successive 200-μl fractions eluted with the same buffer containing 1 mM GalNAc-4-SO₄ (E1, E2, and E3) were analyzed by SDS-PAGE. The SorLA was detected using rabbit anti-SorLA 6.7 following electrophoretic transfer to PVDF membranes.

**FIGURE 6. Recombinant SorLA/LR11 co-expressed with either mouse β₁,4GalNAcTIII or β₁,4GalNAcTIV is modified with terminal GalNAc.** Recombinant SorLA/LR11 was expressed in CHO cells alone (A) or in combination with β₁,4GalNAcT from C. elegans (B), β₁,4GalNAcTIII (C), or β₁,4GalNAcTIV (D). The SorLA/LR11 was solublized with Triton X-100 and incubated with WFA-agarose. Four percent of the extract (T), the WFA unbound fractions (UB), and each of three wash fractions (W1, W2, and W3), and 40% of three fractions eluted with 50 mM GalNAc (E1, E2, and E3) were analyzed by SDS-PAGE. Intact SorLA/LR11 (solid arrow) was identified using rabbit anti-SorLA 6.7 directed against a fibronectin type III domain. Additional species seen in the elution fractions reflect components in the elution buffer that are non-specifically labeled.
to modify all of the terminal β1,4-linked GalNAc present on recombinant SorLA/LR11 when the latter is expressed in HEK-293 cells.

Recombinant β1,4-Acetylgalactosamine Transferases Modify Oligosaccharides on SorLA/LR11 with GalNAc—Three β1,4-A-acetylgalactosamine transferases (β1,4GalNAcT) have been described that are able to modify N-linked oligosaccharides with β1,4-linked GalNAc: a β1,4GalNAcT cloned from Caenorhabditis elegans (39) (β1,4GalNAcT-CE), and two β1,4GalNAcTs, β1,4GalNAcTIII and β1,4GalNAcTIV, cloned from human cDNA libraries (40, 41). We cloned the mouse orthologues of β1,4GalNAcTIII and β1,4GalNAcTIV and established that they are active using both in vitro and in vivo assays. CHO cells expressing a single copy of β1,4GalNAcT-CE, β1,4GalNAcTIII, or β1,4GalNAcTIV at the same location in the CHO cell genome and under the control of the EF-1α-promotor were prepared using Flp-In CHO cells (Invitrogen) and the pEF5/FRT/V5-D-TOPO expression vector (Invitrogen) as described by the supplier. The expression of each β1,4GalNAcT was confirmed by Western blot analysis using an antibody directed at the V5 epitope located at the carboxyl terminus. Recombinant SorLA/LR11 was expressed in each of these CHO cell lines and tested for the presence of terminal β1,4-linked GalNAc by passage over immobilized WFA-agarose (Fig. 6). SorLA/LR11 expressed in Flp-In CHO cells was not bound by WFA-agarose (A), whereas SorLA/LR11 expressed in Flp-In CHO cells expressing β1,4GalNAcT-CE, β1,4GalNAcTIII, or β1,4GalNAcTIV was in each case bound by WFA-agarose (filled arrows in B–D). Thus, each of the three known β1,4GalNAcTs is capable of modifying oligosaccharides on SorLA/LR11 with terminal GalNAc.

The Vps10p Domain of SorLA/LR11 Contains Recognition Determinants That Are Required for GalNAc Addition to its N-Linked Oligosaccharides—The amino-terminal 755 amino acids of SorLA/LR11 contain the Vps10p domain and five potential N-linked glycosylation sites (see Fig. 7). Replacement of the carboxyl-terminal 1460 amino acids of SorLA/LR11 with a V5 epitope followed by six His residues yields a glycoprotein, SorLA/LR11-V5His, that is secreted into the medium. When expressed in parent Flp-In CHO cells that do not contain any β1,4GalNAcT activity, SorLA/LR11-V5His is not bound by either immobilized Cys-Fc (not shown) or WFA-agarose (Fig. 8, Wt CHO). In contrast, SorLA/LR11-V5His expressed in HEK-293 cells containing both β1,4GalNAcT and GalNAc-4-sulfotransferase activity is bound by both immobilized Cys-Fc (not shown) and WFA-agarose (Fig. 8, Wt 293T). Thus, the Vps10p domain contains N-linked oligosaccharides and one or more recognition determinants that are capable of mediating the selective addition of β1,4-linked GalNAc by the protein-specific β1,4-GalNAcTs that are expressed in HEK-293T cells. These β1,4-GalNAcTs modify the oligosaccharides on glycoproteins such as LH (21, 35), tissue factor pathway inhibitor (34), and protein C (42). In the case of the α-subunit of the glycoprotein hormones, we have shown that basic amino acids in the sequence Pro-Leu-Arg-Ser-Lys-Lys are components of the recognition determinant utilized by the β1,4-GalNAcTs in the pituitary (43, 44). Three distinct clusters of basic amino acids are present within the Vps10p domain of SorLA/LR11 (see Fig. 7), each of which could serve a recognition determinant for GalNAc addition. The potential recognition sequences were deleted or mutated in the SorLA/LR11-V5His construct as illustrated in Fig. 7, and the constructs were expressed in HEK-293T cells. Only those mutants, Mu-3 and Mu-5, in which the furin cleavage site, KPLRRKR, was deleted and the sequence KTVFQQ was changed to KTVFKRR were completely devoid of GalNAc following expression in HEK-293T cells (Fig. 8). Thus, either KPLRRKR or KTFRKKR but not RRTRGKR are able to mediate recognition by the protein-specific β1,4-GalNAcTs in HEK-293T cells. The same experiments were performed with Flp-In CHO cells expressing β1,4-GalNAcTIII or β1,4-GalNAcTIV (not shown) with the same results as summarized in Table 2, indicating that these two transferases display similar specificities with respect to GalNAc addition to SorLA/LR11.

DISCUSSION
The addition of terminal β1,4-linked GalNAc-4-SO₄ to N-linked oligosaccharides is a highly regulated and specific
process. We have shown that expression of a glycoprotein containing a peptide sequence that is recognized by a protein-specific \( \beta \)-GalNAcT, a protein-specific \( \beta \)-GalNAcT, and a GalNAc-4-sulfotransferase are all required for terminal GalNAc-4-SO\(_4\) addition (45). In the case of the glycoprotein hormones, we have determined that the peptide recognition determinant, protein-specific \( \beta \)-GalNAcT activity, and GalNAc-4-sulfotransferase activity have been conserved throughout vertebrate evolution. As a consequence glycoprotein hormones of all vertebrates from fish to man bear this unique sulfated carbohydrate structure (45, 46). The terminal GalNAc-4-SO\(_4\) is recognized by the Man/GalNAc-4-SO\(_4\) receptor in hepatic endothelial cells. This receptor controls the circulatory half-life of LH following its secretion into the blood (21–25, 47). Thus, this unique sulfated carbohydrate structure has important functional consequences for glycoproteins that are secreted into the blood.

We have sought to identify additional glycoproteins that bear this uncommon sulfated carbohydrate structure, to determine whether terminal GalNAc-4-SO\(_4\) moieties also have functional consequences for these glycoproteins. Cys-Fc immunostains of histologic sections from different tissues have revealed specific structures and cells containing glycoproteins that potentially terminate with \( \beta \)-1,4-linked GalNAc-4-SO\(_4\). The presence of protein-specific \( \beta \)-1,4-GalNAcT (40, 41) and GalNAc-4-sulfotransferase (38, 48–50) activity and/or mRNA in these same locations indicates that one or more glycoproteins bearing terminal GalNAc-4-SO\(_4\) are present. SorLA/LR11, like sialoadhesin and CD45 that are also reported to bear terminal GalNAc-4-SO\(_4\), is an integral membrane protein rather than a circulating hormone such as LH that also bears terminal GalNAc-4-SO\(_4\). It is, therefore, highly unlikely that the SorLA/LR11 bearing GalNAc-4-SO\(_4\) from either the kidney or the brain will ever encounter the Man/GalNAc-4-SO\(_4\) receptor expressed by hepatic endothelial cells. Yet, SorLA/LR11 synthesized by two unrelated cell types, principal cells of the kidney (31) and neurons in the central nervous system (1, 2), is in both instances modified with terminal \( \beta \)-1,4-linked GalNAc-4-SO\(_4\). It is therefore likely that the terminal \( \beta \)-1,4-linked GalNAc-4-SO\(_4\) present on SorLA/LR11 will prove to have important functional consequences.

Based on the mutations we have introduced into the Vps10p domain of SorLA/LR11, there are two clusters of basic amino acid residues that are able to act as recognition determinants for the \( \beta \)-1,4GalNAcT activities present in HEK-293T cells and for both recombinant \( \beta \)-1,4GalNAcTIII and \( \beta \)-1,4GalNAcTIV when expressed in CHO cells. We have found that \( \beta \)-1,4GalNAcTIII and \( \beta \)-1,4GalNAcTIV are also both able to modify the N-linked oligosaccharides on the glycoprotein hormone \( \alpha \) subunit,\(^3\) indicating they have similar specificities with respect to the peptide recognition determinant. The presence of GalNAc-4-SO\(_4\) on the N-linked oligosaccharides in the Vps10p domain suggests that the presence of this sulfated carbohydrate may modulate the interaction of this domain with other proteins. The \( \mathrm{NH}_2 \)-terminal propeptide released from

\[^3\]D. Fiete, Y. Mi, E. L. Oats, M. C. Beranek, and J. U. Baenziger, manuscript in preparation.

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**TABLE 2**

| Bound by WFA | HEK-293T | CHO/GalNAcTIII | CHO/GalNAcTIV |
|-------------|----------|----------------|---------------|
| Wt          | Wild type | +              | +             |
| Mu-1        | +         | ND\(^{a}\)      | ND            |
| Mu-2        | +         | ND             | ND            |
| Mu-3        | +         | +              | +             |
| Mu-4        | +         | +              | +             |
| Mu-5        | -         | -              | -             |

\(^{a}\) ND, not determined.

**FIGURE 8. Analysis of Vsp10p domain mutants.** The Vsp10p domain (Wt) and mutants with alterations in the sequences of three different regions rich in basic amino acids, Mu1–Mu6, as illustrated in Fig. 7 were expressed in HEK-293T cells. The medium containing the Vsp10p domain was incubated with WFA-agarose and any bound Vsp10p domain eluted with 1 mM GalNAc. Aliquots of the medium, the medium following incubation with WFA-agarose, the wash fractions, and the bound material eluted with GalNAc were analyzed by Western blot using antibody directed against the V5 epitope following SDS-PAGE and electrophoretic transfer to PVDF. Equal percentages of material were analyzed from the medium, unbound fraction, and eluted (bound) fractions except for 1 where 4-fold more of the bound fraction was analyzed than the total or unbound fraction and 2 where 10-fold more of the bound fraction was analyzed that the total or unbound fraction.

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**Post-translational Modification of SorLA/LR11**

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The Vps10p domain of SorLA/LR11 was expressed in HEK-293T cells, CHO/GalNAcTIIIi, and CHO/GalNAcTIV cells. The medium was collected and incubated with WFA-agarose. After washing the WFA-agarose, bound material was eluted with 50 mM GalNAc. The amount of the Vps10p domain present in the medium, unbound fraction, washes, and eluted fractions were determined by Western blotting with anti-V5 following SDS-PAGE and electrophoretic transfer to PVDF. Forms of the Vps10p domain that were bound are indicated with a plus (+), those that were not bound with a minus (−). Insufficient levels of Mu-2 were released into the medium for analysis.
SorLA/LR11 by furin mediated cleavage, as well as the peptides neurotensin, and the hydrolase head activator peptide, are each able to bind to the Vps10p domain of SorLA/LR11. In contrast, apolipoprotein E and lipoprotein lipase are thought to bind to other regions of the luminal domain of SorLA/LR11 such as the LDL receptor repeats (6). Thus, the presence or absence of terminal GalNAc-4-SO₄ may selectively modulate the binding of ligands such as propeptide or neurotensin to the Vps10p domain of SorLA/LR11.

The function of SorLA/LR11 in either the kidney or the brain has not yet been established. SorLA/LR11 is able to mediate endocytosis of ligands such as apoE; however, only 10% of the receptor is expressed at the cell surface (6). The cytosolic domain of SorLA/LR11 contains a motif that results in binding to proteins GGA-1 and GGA-2 (Golgi-localized, γ-ear containing, ARF binding). GGA proteins are involved in Golgi-endosome sorting and SorLA/LR11 is found predominantly on intracellular vesicular membranes (3). Thus, SorLA/LR11 is thought to play a role in intracellular trafficking.

Recent studies have shown that the luminal domain of SorLA/LR11 binds amyloid precursor protein and reduces the rate of amyloid precursor protein cleavage to amyloid β-peptide (Aβ). Increased levels of Aβ are present in the brains of mice that do not express SorLA/LR11 (51). Furthermore, reduced levels of SorLA/LR11 are found in brains of Alzheimer disease patients (52). Similarly, it is possible that SorLA/LR11 in the principal cells of the kidney influences the intracellular trafficking of intracellular shuttling compartments that contain aquaporin2. The vasopressin-mediated trafficking of aquaporin2 between intracellular compartments and the plasma membrane is important for the maintenance of water balance (31, 53). The presence or absence of terminal GalNAc-4-SO₄ has the potential to modulate binding of a number of different proteins with SorLA/LR11 and thereby alter their intracellular trafficking. This would only be the case for the subset of SorLA/LR11 that is modified with terminal GalNAc-4-SO₄. At present we do not know what determines the proportion of SorLA/LR11 that is modified with GalNAc-4-SO₄. Not all cells that express SorLA/LR11 necessarily also express the β₄GalNAcT and GalNAc-4-sulfotransferase activities required for GalNAc-4-SO₄ addition. Alternatively, the extent of modification within individual cells may be regulated, producing two distinct populations of SorLA/LR11 that differ in the patterns of terminal glycosylation.

SorLA/LR11 is one of a small number of integral membrane glycoproteins, including sialoadhesin and CD45 (20), selectively modified with terminal β₄-linked GalNAc-4-SO₄ in vivo. This is true in at least two distinct cell types, distal collecting tubules in the kidney and neurons in specific regions of the central nervous system. Because the addition of β₄-linked GalNAc-4-SO₄ is protein-specific and highly regulated and only a fraction of SorLA/LR11 is modified with this structures, the presence of this sulfated carbohydrate structure may be critical for some aspects of SorLA/LR11 function. Determining the functional significance of these sulfated carbohydrates on SorLA/LR11 may provide insight into the function of this complex receptor.

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