N-glycosylation and topology of the human SLC26 family of anion transport membrane proteins

Jing Li, Fan Xia, and Reinhart A. F. Reithmeier

Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

Submitted 30 January 2014; accepted in final form 13 March 2014

Li J, Xia F, Reithmeier RA. N-glycosylation and topology of the human SLC26 family of anion transport membrane proteins. Am J Physiol Cell Physiol 306: C943–C960, 2014. First published March 19, 2014; doi:10.1152/ajpcell.00030.2014.—The human solute carrier (SLC26) family of anion transporters consists of 10 members (SLC1A1–11, SLC1A10 being a pseudogene) that encode membrane proteins containing ∼12 transmembrane (TM) segments with putative N-glycosylation sites (-NXS/T-) in extracellular loops and a COOH-terminal cytosolic STAS domain. All 10 members of the human SLC26 family, FLAG-tagged at the NH2 terminus, were transiently expressed in HEK-293 cells. While most proteins were observed to contain both high-mannose and complex oligosaccharides, SLC26A2 was mainly in the complex form, SLC26A4 in the high-mannose form, and SLC26A8 was not N-glycosylated. Mutation of the putative N-glycosylation sites showed that most members contain multiple N-glycosylation sites in the second extracytosolic (EC) loop, except SLC26A2, which was N-glycosylated in EC loop 4. Immunofluorescence staining of permeabilized cells localized the proteins to the plasma membrane and the endoplasmic reticulum, with SLC26A11, which was N-glycosylated in EC loop 4. Immunofluorescence staining of permeabilized cells localized the proteins to the plasma membrane and the endoplasmic reticulum, with SLC26A2 highly localized to the plasma membrane. N-glycosylation was not a necessary requirement for cell surface expression as the localization of nonglycosylated proteins was similar to their wild-type counterparts, although a lower level of cell-surface biotinylation was observed. No immunostaining of intact cells was observed for any SLC26 members, demonstrating that the NH2-terminal FLAG tag was located in the cytosol. Topological models of the SLC26 proteins that contain an even number of transmembrane segments with both the NH2 and COOH termini located in the cytosol and utilized N-glycosylation sites defining the positions of two EC loops are presented.

THE SLC26 FAMILY OF ANION transporters is found in all kingdoms of life indicating a fundamental importance of these membrane proteins in biology. The human SLC26 family (Table 1) of anion transporters consists of 10 members (SLC26A1-11, A10 being a pseudo gene) (18, 36, 62, 63). The human SLC26 proteins contain ∼750 residues but range in size from 606 residues (SLC26A11) to 970 residues (SLC26A8). The proteins all consist of two major domains: a NH2-terminal membrane domain with ∼12 putative transmembrane (TM) segments and a COOH-terminal STAS (sulfate transporter antagonist of anti-sigma factor) domain located in the cytosol (53). Structures of the STAS domain of several bacterial members of the SLC26 family have been obtained (4, 54), as well as the structure of a modified version of the STAS domain of rat SLC26A3 protein (40). All of the core structures resemble the Bacillus anti-sigma factor SpoIIAA, a 110 amino acid phospho-protein with an alpha/beta topology consisting of a central four-stranded beta sheet sandwiched between three peripheral alpha helices (2, 52). The STAS domain of the sole Escherichia coli member of the SLC26 family, YchM, was isolated as a complex with acyl carrier protein (ACP), suggesting a link between anion transport and fatty acid metabolism (4). This protein has been characterized as a dicarboxylic acid (e.g., succinate and fumarate) transporter active at low pH and has been named DauA (23). In contrast to the STAS domain there is little structural information available for the membrane domain of the SLC26 family of transporters. A study of the topology of BicA, a family member found in photosynthetic cyanobacterium Synechococcus (43), is consistent with a 12 TM model, with both NH2 and COOH termini located in the cytosol (42, 58).

The SLC26 family members (Table 1) typically catalyze chloride/bicarbonate anion exchange across the apical or basolateral membranes of polarized cells found in organs like the stomach, intestine, kidney, pancreas, lung, and inner ear, although some are associated with anion channel activity (18, 36, 38). For a recent review on SLC26 family members, see also Alper and Sharma (1). SLC26A1 and A2 transport sulfate, oxalate, and glyoxalate in an exchange mode with chloride (6, 19, 37, 45, 49, 50, 68). SLC26A1 (Sat-1) is located in the basolateral membrane of hepatocytes, enterocytes, and proximal tubular epithelial cells, while SLC26A2 (DTDTST) is located in chondrocytes and in the apical membrane of colonic epithelial cells and renal proximal cells. SLC26A3 (DRA/CLD) is a well-characterized chloride/bicarbonate exchanger present in the apical membrane of intestinal enterocytes where it works in conjunction with the NHE3 Na+/H+ exchanger to mediate electroneutral NaCl reabsorption by the intestine (10, 33, 51, 55). In addition to bicarbonate and chloride, SLC26A4 (pdracin) can also transport iodide, which may play an important role in the thyroid (12, 46, 56, 57). SLC26A5 (prestin) acts as a voltage-sensitive chloride sensor in the lateral membrane of the cochlear outer hair cell (70). SLC26A6 (PAT-1) is widely expressed and has a broad set of substrates (30, 55, 68). SLC26A7 is a basolateral protein in gastric parietal cells and in renal type A intercalated cells (18, 31) and acts as a chloride channel (26). SLC26A8 is expressed in sperm, and mutations result in male infertility (15, 31, 64). SLC26A9 has a wide-spread distribution but is abundant in the apical membrane of epithelial cells in the lung, brain, and stomach where it mediates chloride/bicarbonate exchange or acts as a chloride and bicarbonate channel in concert with cystic fibrosis transmembrane regulator (CFTR) (17, 31). SLC26A10 is a pseudogene (36). SLC26A11 may serve as a lysosomal sulfate transporter, and it is reported to facilitate chloride channel activity (66).

Mutations in the SLC26 genes (Table 1) are linked to a number of inherited human diseases (1), most notably in...
SLC26A2 (47), A3 (67), A4 (8, 13, 39, 65), and recently in A8 (15). These mutations in the membrane or STAS domain cause a defect in functional expression of the protein, often due to trafficking defects and retention of the protein in the endoplasmic reticulum (ER). Expression of SLC26A2 with mutations (R279W, Q454P, C653S, and A715V) in HEK cells resulted in diminished protein expression and transport activity relative to the wild-type (WT) protein, while the H9004V340 and G678V mutations resulted in intracellular retention (24). Transport defects were also noted for the R279W and A386V mutants when expressed in Xenopus oocytes, with A386V having reduced cell surface expression but R279W being expressed at normal levels (21). Mutations (e.g., I544N) in the STAS domain of SLC26A3 linked to chloride-losing diarrhea-induced misfolding of the domain and complete retention of the protein in the ER (16). Similarly, mutations in the STAS domain (e.g., H723R) of membrane domain of SLC26A4 that result in Pendred syndrome cause ER retention of the mutant protein (69) or functional defects (11). Mutations (R87Q, E812K, and R954C) in the testis-specific SLC26A8 resulted in a decreased stability of the protein and impaired its ability to activate CFTR (15). Mutation of Asp107 in plant SHST1, which is conserved throughout the SLC26 family (e.g., Asp74 in human SLC26A3) resulted in an inactive transporter, but the mutation did not affect its cell surface expression in yeast (28). Introducing mutations that cause DTDST in humans due to defects in SLC26A2 in equivalent sites in SHST1 caused functional defects (25, 32). A cysteine-less mutant of SHST1 was fully functional (22) permitting cysteine-scanning mutagenesis experiments to be performed (71). Mutation of the STAS domain of arabidopsis sulfate transporter SULTR1.2 resulted in trafficking and functional defects (48, 60, 61). This domain binds the enzyme cysteine synthase (59) coordinating

Table 1. N-glycosylation sites in the human SLC26 family of anion transporters

<table>
<thead>
<tr>
<th>SLC26 Family Member (Protein Name and NCBI Accession Number, Variant)</th>
<th>Protein Residues and Molecular Mass1</th>
<th>Link to Inherited Human Diseases</th>
<th>N-Glycosylation Sites2</th>
<th>Distribution and Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC26A1 (Sat-1 NM_213613.2, variant 3)</td>
<td>701 aa 75,016 Da</td>
<td>Congenital chloride-losing diarrhea (CCD/CLD)</td>
<td>Asn158* (EC2) Asn163* (EC2)</td>
<td>Liver, intestine, kidney, pancreas Basolateral sulfate, oxalate/Cl⁻ exchanger</td>
</tr>
<tr>
<td>SLC26A2</td>
<td>739 aa 81,662 Da</td>
<td>Diastrophic dysplasia</td>
<td>Asn199* (EC2) Asn205* (EC2)</td>
<td>Chondrocytes, kidney, intestine, pancreas Apical sulfate, oxalate/Cl⁻ exchanger</td>
</tr>
<tr>
<td>(DTDST NM_000112.2)</td>
<td></td>
<td></td>
<td>Asn357* (EC4) utilized if Asn199 or Asn205 are mutated</td>
<td></td>
</tr>
<tr>
<td>SLC26A3</td>
<td>764 aa 84,305 Da</td>
<td>Congenital chloride-losing diarrhea (CCD/CLD)</td>
<td>Asn153* (EC2) Asn161* (EC2) Asn164* (EC2)</td>
<td>Intestine, sweat gland, pancreas, kidney Apical Cl⁻/HCO₃⁻ exchanger</td>
</tr>
<tr>
<td>(DRA NM_000111.1)</td>
<td></td>
<td></td>
<td>Asn165* (EC2) Asn167* (EC2)</td>
<td>Apical Cl⁻/HCO₃⁻ exchanger Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>SLC26A4</td>
<td>780 aa 85,723 Da</td>
<td>Pendred syndrome (deafness, goiter)</td>
<td>Asn172* (EC2) Asn172* (EC2)</td>
<td>Cochlea, thyroid, kidney (βIC), lung Apical Cl⁻/HCO₃⁻ (I⁻) exchanger</td>
</tr>
<tr>
<td>(Pendrin NM_000441.1)</td>
<td></td>
<td></td>
<td>Asn241 Asn163* (EC2)</td>
<td>Motor protein of outer hair cells of cochlea</td>
</tr>
<tr>
<td>SLC26A5</td>
<td>744 aa 81,264 Da</td>
<td>Deafness</td>
<td>Asn214 Asn163* (EC2)</td>
<td>Ubiquitous, intestine, kidney, pancreas, heart (BLM/PT) Apical Cl⁻/HCO₃⁻ exchanger</td>
</tr>
<tr>
<td>(Prestin NM_198999.1, variant a)</td>
<td></td>
<td></td>
<td>Asn166* (EC2) Asn167* (EC2)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>SLC26A6</td>
<td>758 aa 82,839 Da</td>
<td>Pendred syndrome (deafness, goiter)</td>
<td>Asn172* (EC2) Asn172* (EC2)</td>
<td>Kidney (βIC), parietal Apical Cl⁻/HCO₃⁻, sulfate exchanger</td>
</tr>
<tr>
<td>SLC26A7</td>
<td>663 aa 73,033 Da</td>
<td>Pendred syndrome (deafness, goiter)</td>
<td>Asn125* (EC2) Asn131* (EC2)</td>
<td>Kidney (βIC), parietal Apical Cl⁻/HCO₃⁻, sulfate exchanger</td>
</tr>
<tr>
<td>(NM_134266.1, variant 2)</td>
<td></td>
<td></td>
<td>Asn241 Asn163* (EC2)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>SLC26A8</td>
<td>970 aa 109,006 Da</td>
<td>Male infertility</td>
<td>Asn192 Asn277 Asn384</td>
<td>Endosomes Testis (sperm) Cl⁻/HCO₃⁻, oxalate exchanger</td>
</tr>
<tr>
<td>(Tat-1 NM_0529656.2, variant 1, V73M)</td>
<td></td>
<td></td>
<td>Asn153* (EC2) Asn156* (EC2)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>SLC26A9</td>
<td>791 aa 86,988 Da</td>
<td>Pendred syndrome (deafness, goiter)</td>
<td>Asn294* (EC4)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>(NM_052934.2, variant 1)</td>
<td></td>
<td></td>
<td>Asn294* (EC4)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>SLC26A11</td>
<td>606 aa 65,299 Da</td>
<td>Pendred syndrome (deafness, goiter)</td>
<td>Asn294* (EC4)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
<tr>
<td>(NM_173626.2, variant 2)</td>
<td></td>
<td></td>
<td>Asn294* (EC4)</td>
<td>Apical Cl⁻/HCO₃⁻, formate, oxalate exchanger</td>
</tr>
</tbody>
</table>

EC, extracytosolic.1Taken from GeneCard (http://www.genecards.org) for the human SLC26 family.2Putative N-glycosylation sites that contain the consensus NXST sequon within extracellular loops in the membrane domain, excluding those within the cytosolic NH₂-terminal region and the COOH-terminal STAS domain.
sulfate uptake and metabolism, thereby creating a membrane transport metabolon (35).

Figure 1 shows the location of potential N-glycosylation sites (-NXS/T-) in a sequence alignment of the membrane domains of the various members of the SLC26 family. Most family members have potential N-glycosylation sites within a poorly conserved region located at positions similar to Asn158 and 163 in SLC26A1 (Table 1). SLC26A11 contains a deletion in this region but has an acceptor site downstream at Asn294. Kyte-Dalrymple analyses of the amino acid sequences predicted that the SLC26 family members had 12 TM segments with a COOH-terminal STAS domain. Most family members contain putative N-glycosylation sites in EC loop 2. Consensus N-glycosylation sites (-NXS/T-) within the hydrophilic COOH-terminal STAS domain, which starting around residue 500, were not considered in this study as they are cytosolic and would not be utilized.

Limited studies have been performed on the N-glycosylation status of members of the human SLC26 family and the effect of this modification on protein folding, trafficking, and functional expression. The nature and level of processing of the N-glycan from high-mannose to complex oligosaccharide provide valuable information concerning the ability of these membrane glycoproteins to exit the ER and traffic to the plasma membrane. Localization of utilized N-glycosylation sites provides topological markers for extracytosolic (EC) regions of membrane proteins, essential to develop accurate folding models. The role of the N-linked oligosaccharide in the functional expression of these membrane proteins can be determined by mutation of the acceptor (-NXS/T-) sites. Previous studies of N-glycan sites have focused on a few members of the SLC26 family. SLC26A2 (DTDST) expressed in HEK cells showed two bands of 120 and 80 kDa with the upper band predominating (24, 37), as did the protein in isolated rat proximal tubule microvillus membrane vesicles (9). Previous N-glycosylation studies of SLC26A3 identified N-glycosylation sites at closely spaced positions Asn153, 161, and 165, showing that this loop region is extracellular (20). Furthermore, N-glycosylation promoted cell surface expression and afforded protection against proteolysis of this family member (20). Pendrin (SLC26A4) is N-glycosylated, and mutations linked to disease affected its trafficking and N-glycosylation pattern (69). Prestin (SLC26A5) is N-glycosylated at Asn163 and 166; however, N-glycosylation was not required for its cell surface expression but had some effect on its electrophysiological properties (34, 44).

In this study we expressed all 10 members of the human SLC26 family in HEK-293 cells to directly compare their N-glycosylation patterns in the same cell system and to determine the role of N-glycosylation in trafficking of this family of membrane proteins to the cell surface. Identification of N-glycosylation sites through mutagenesis localized the N-glycosylated sites within EC loop 2 in most family members (SLC26A1, A2, A3, A4, A5, A6, A7, and A9). SLC26A11 contained a single site of N-glycosylation in EC loop 4, a loop that is larger in this protein than other family members (Fig. 1), while SLC26A8 was not N-glycosylated in transfected HEK cells. SLC26A2 had a cryptic N-glycosylation site in EC loop 4 that was utilized when one or both of the sites in EC loop 2 were mutated. The use of NH2- and COOH-tagged versions of the proteins provided topological information showing that both the NH2 and COOH termini are cytosolic, while the utilized N-glycosylation sites identified the locations of EC loops 2 and 4.

MATERIALS AND METHODS

DNA construction of WT and nonglycosylated SLC26 mutants.
Original cDNAs coding SLC26A1, A2, A3, A4, A5, A7, and A11 with COOH-terminal myc-DDK tag on plasmid pCMV6-Entry vector were purchased from Origene Technologies (Rockville, MD) and were used in these studies. The cDNAs encoding SLC26A2, A3, A6, and A9 in pGEMHE were obtained from Dr. M. F. Romero (Mayo), and the plasmids for SLC26A6 and A9 were used in these studies. The cDNA coding SLC26A8 in pcDNA3 (pRK5) vector was a gift from Dr. Gérard Gacon (Paris, France). These cDNAs encoding human SLC26 A1–11 WT, except A10, a pseudogene, were amplified by PCR using PfuUltra II Fusion High-Fidelity DNA polymerase (Agilent Technologies) and inserted into plasmid vector pcDNA3 (Invitrogen, Life Technologies) at NotI sites at both 5’- and 3’-ends. A new NotI site encoding three alanines was created just before the first methionine of SLC26A1, A2, A4, A5, A7, A8, and A11 to facilitate subcloning. A consecutive triple FLAG (DYKDDDDK) tag was created by PCR subcloning method at the NH2-terminus of all SLC26 family members. These clones did not include the COOH-terminal myc-DDK tag originally present in the Origene plasmids. Nonglycosylated mutants (N0) were created by site directed mutagenesis using Q5 site-directed mutagenesis kit (New England Biolabs) by mutating asparagine to aspartate (NxyxD, where x = the residue number mutated) in individual N-glycosylation acceptor sites (-NXS/T- and then in various combinations. All primers were synthesized by Integrated DNA Technologies. All DNA constructs were confirmed by DNA sequencing by ACGT (Toronto, Canada).

Cell culture. Human embryonic kidney (HEK-293) cells and Madin-Darby canine kidney (MDCK) cells were grown either in DMEM or DMEM-F12 medium supplied with 10% fetal bovine serum (FBS), 1% penicillin, and 0.5% streptomycin (GIBCO, Life Technologies) under 5% CO2 at 37°C.

Transfection and expression in HEK-293 cells. HEK 293 cells growing in sixwell plates were transfected using Lipofectamine LTX and Plus Reagent (Invitrogen, Life Technologies), with 1 µg plasmid DNA per well. Cells were harvested 24–48 h after transfection.

Transfection and expression in MDCK cells. MDCK cells grown in sixwell plates were transfected using GenJet In Vitro DNA Transfection Reagent for MDCK cells (SignaGen Laboratories, Rockville, MD), with 2 µg plasmid DNA per well. The MDCK cells were harvested 30 h later and analyzed for SLC26 protein expression by immunoblotting. For establishing a stable MDCK cell line, the cells were trypsinized and transfected to a T25 flask containing 0.1 mg/ml G418 (GIBCO, Life Technologies). Resistant cells continued growing by providing fresh media containing G418 every 2 days. After antibiotic selection for 7 days, the surviving MDCK cells were grown in media containing 0.5 mg/ml G418. Three weeks after transfection, the cells were harvested for immunoblotting.

Immunoblotting (Western blotting). Transfected HEK cells grown for 48 h in sixwell plates were lysed in 0.5 ml PBS containing 1% C12E8 (octaethylene glycol mono-n-dodecyl ether; NIKKO Chemicals, Japan) with protease inhibitors: 2 µM leupeptin, 0.3 µM aprotonin, 1.5 µM peptatin A (Bioshop, Burlington, ON, Canada), and 1 mM PMSF (Sigma-Aldrich) on ice for 30 min and then centrifuged at 17,000 × g for 30 min at 4°C to remove cell debris. The supernatant was mixed with the same volume of 2× Laemmli sample buffer containing 4% sodium dodecyl sulfate (SDS) at room temperature. Proteins were resolved by 8% SDS-PAGE and transferred onto nitrocellulose membrane. SLC26 proteins were detected by mouse monoclonal anti-FLAG M2 antibody (1:5,000 dilution; Sigma-Aldrich), followed by horseradish peroxidase (HRP; 1:5,000 dilution; Cell Signaling Technology, Danvers, MA). The immunoblot was developed using horseradish peroxidase (HRP; 1:5,000 dilution; Cell Signaling Technology, Danvers, MA). The immunoblot was developed using enhanced chemiluminescence (ECL) reagent (Roche, Indianapolis, IN).
**N-Glycosylation of SLC26 Anion Transporters**

A1 QVDREQLAQFD--PSQDGLQPGNNSLTLGSGAALDCDRCDYAIRVATALTLMGTYQ 192
A2 EVDRARLQAGYUNASHAPSFLMGSVNNGLLNHDSAPRCDAYCAIMVGVSTPEILAVYQ 233
A3 LAVSGAVSKVAPD----RFLGKLGNNNNSLD--DERRVVAARASSTVLSQSIQ 190
A4 ----SVVNSMAPO----EHFLVSSSSSNVLMTIDMTAARDTARVFMLVCIQ 200
A5 GVAVRIVPDDVI--PG--------GVNATGEMPADAARLGBKVAMSTLISQSIQ 190
A6 SVTSELAPQAI ND------S----MINEFM--ARDAAVYQQASTLSVLQYQ 184
A7 ------AVERVIPY---QN----MCLNLTQNSTVSLGDSRESNRLHVAAWVAPLSVQG 159
A8 N-----VLKVSFPN----NG-QGLVQFSVFKNSFAASPYLMGDYKSLSWAVTTPLTGIIQ 209
A9 NICLQLAPESKFQ----VFNP----------MNTQKIVYDAAEMLHPSLACITQI 184
A11 ------------------------FYTFHEPAVYALLFSLQSCIQ 134

**Fig. 1.** Protein sequence alignment of human SLC26 family members using ClustalW. Utilized N-glycosylation sites (-NXS/T-) are colored in green. Nonutilized N-glycosylation sites are colored in magenta and yellow (cryptic site). Hydropathy analyses predicted that the SLC 26 family members consist of 12 transmembrane segments placing the cluster of N-glycosylation sites in extracytosolic (EC) loop 2.

**References**

C946
vers, MA). Then, endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected on the same blot using a mouse anti-GAPDH antibody (1/10,000 dilution; EMD Millipore, Billerica, MA), 500 U of peptide N-glycosidase F (PNGase F; New England Biolabs), or in buffer alone (control) and incubated at 37°C for 1 h, followed by mixing with the same volume of 2X Laemmli sample buffer containing 4% sodium dodecyl sulfate (SDS). SLC26 protein was resolved by 8% SDS-PAGE and detected by mouse monoclonal anti-FLAG M2 antibody using Western blotting.

**Cell surface biotinylation.** Twenty-four posttransfected HEK 293 cells grown on a sixwell plate were washed by ice-cold borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl, and 1.8 mM CaCl2) and incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich) to block nonspecific binding for 30 min followed by monoclonal primary antibody of mouse anti-FLAG M2 (1:1,000 dilution) for 45 min and then a secondary antibody of goat anti-mouse IgG Alexa Fluor 488 (green) conjugated or donkey anti-rabbit IgG-cy3 (red) conjugated (1/500 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. To detect total expressed protein, after fixation, permeabilization, and BSA blocking, the cells were incubated with mouse anti-FLAG M2 or rabbit anti-calnexin (1/200 dilution; Cell Signaling Technology) for 45 min followed by goat anti-mouse IgG Alexa Fluor 488 (green) conjugated donkey anti-rabbit IgG-cy3 (red) conjugated (1/500 dilution; Jackson ImmunoResearch Laboratories) for 30 min, respectively. Antibody incubation buffer and wash buffer were PBS (pH 7.4) with 1 mM CaCl2 and 1 mM MgCl2. Antibody incubation buffer contained 1% BSA. Labeled cells were preserved by Fluorescence Mounting Medium (DAKO). The stained cells were observed using a Laser Scanning LSM510 microscope (Carl Zeiss Microscopy).

**Protein intensity, sequence alignment, hydropathy plot, and topology prediction.** Protein band intensities on immunoblots in the linear range of samples from at least three separate cell transfections were quantified using the National Institutes of Health (NIH) ImageJ program (http://rsweb.nih.gov/ij/). Colocalization of proteins on confocal fluorescent images of transfected cells was quantified using NIH ImageJ JACoP plugin software (http://rsweb.nih.gov/ij/plugins/track/jacop.html). Protein sequence alignment analysis was obtained by webware ClustalW program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_clustalw.html). Kyte-Doolittle hydropathy plots to predict the location of hydrophobic transmembrane segments were constructed using the ExPASy program (http://web.expasy.org/cgi-bin/protscal/protscal.pl). The transmembrane topology oligonucleotide of each member of the SLC26 family was predicted by PRODIVE-TMHMM method using the TOPCONS program (http://topcons. cbr.su.se/). Protein 2D topology diagrams were drawn by Protter program (http://wlab.ethz.ch/protter/start/).

### RESULTS

**N-glycosylation of human SLC26 family members.** The first aim of this study was to determine the N-glycosylation pattern of all 10 human members of the SLC26 family of anion transporters. To do this, family members (SLC26A1-11, A10 being a pseudogene) were tagged at their NH2 termini with a triple FLAG epitope tag and transiently expressed in HEK-293 cells. Cells were solubilized in detergent (1% C12E8), and the proteins were then resolved by SDS polyacrylamide gel electrophoresis. Figure 2 shows an immunoblot of the expressed SLC26 proteins detected as FLAG-tagged constructs. The first thing to note is that most of the SLC26 proteins ran as multiple bands at ~100 kDa, typically with a sharp lower molecular mass band and a broader upper band. Exceptions were SLC26A4 and A8, which ran as single sharp bands. SLC26A2 ran on SDS gels as a major upper band with little if any lower band. The lower bands of SLC26A1, A6, A9, and A11 tended to be broader than SLC26A3, A5, and A7; this may be due to higher expression levels and overexpression on this particular immunoblot.

Secondly, the amount of protein detected by immunoblotting, normalized to the expression of GAPDH, varied for the different constructs even though the same amount of cDNA was used in parallel transfection experiments. Quantification of at least three separate transfection experiments of immunoblots of the intact glycoproteins and the nonglycosylated products following N-glycanase F treatment (see below) were performed (Table 2). SLC26A6 expressed at the highest level and was set as 100%. SLC26A11 also expressed very well (65%), while A1, A2, and A9 were at approximately one-third the level of A6. The levels of A3, A4, and A5 were lower still (10–20%), while A8 (3%) was very poorly expressed.
expressed. The differences in expression levels were reproducible and were unlikely due to different immunoreactivity of the constructs as they all had the identical NH2-terminal FLAG tag. Alternatively, the different expression levels may reflect differences in the cellular stability of the SLC26 proteins.

As mentioned above, the SLC26 proteins appeared as multiple bands, typically as a sharp lower band and a broader upper band. Enzymatic deglycosylation of the SLC26 family members was carried out with endoglycosidase H, which cleaves high-mannose oligosaccharides, and N-glycanase F, which cleaves all N-linked oligosaccharides. The lower band was sensitive to endoglycosidase H and contained high-mannose oligosaccharide while the upper bands were resistant to endoglycosidase H treatment (Fig. 3). N-glycanase F collapsed the multiple bands into a single band that represents the mobility of the nonglycosylated protein. Even upon deglycosylation, the proteins (SLC26A1, A2, A6, A7, A9, and A11) often ran as broad bands, suggesting that the apparent heterogeneity was not due to difference in N-glycosylation. The complex bands represent protein that had exited the ER and reached the Golgi or beyond likely to the plasma membrane. SLC26A4 was entirely sensitive to endoglycosidase H, showing that this protein contain high-mannose oligosaccharide that was not processed to complex form in HEK cells. SLC26A8 was not sensitive to deglycosylation by endoglycosidase H or N-glycanase F, suggesting that this family member is unique in not being N-glycosylated.

The percentage of protein with complex oligosaccharide varied among family members (Table 2). SLC26A2 contained over 80% complex oligosaccharide. This indicates that SLC26A2 was processed very efficiently in HEK cells and had moved from the ER to the Golgi or beyond. SLC26A3, A5, A6, and A7 were processed to over 50% complex oligosaccharide, while A1, A9, and A11 were processed to approximately one-third complex. In contrast, SLC26A4 (pendrin) contained only high-mannose oligosaccharide, indicating poor processing and, perhaps, an ER localization. The presence of high-mannose oligosaccharide on SLC26A4 was shown by a lectin shift assay (41) in which concanavalin A was included in the stacking portion of the polyacrylamide gel. This lectin binds high-mannose glycoproteins, slowing their migration and allowing separation from closely spaced high-mannose and nonglycosylated protein. SLC26A4 expressed in HEK cells is in a high-mannose form that shifts and as a nonglycosylated form that does not (Fig. 3). As mentioned above, SLC26A8 was not N-glycosylated and thus does not contain any high-mannose or complex oligosaccharide. There was no change in the mobility of this protein upon treatment with endoglycosidase H or N-glycanase F (Fig. 3), even in a lectin shift assay (data not shown), confirming the lack of N-linked oligosaccharide on SLC26A8.

The SLC26 family members were also transiently expressed in MDCK cells. SLC26A1, A4, A6, A9, and A11 could be detected on immunoblots 30 h after transfection (Fig. 4). As in HEK cells, SLC26A6 was the most highly expressed family member with the majority in the complex form. The pattern of N-glycosylation of SLC26A1 and A11 was also similar to that observed in transfected HEK cells with two major bands and about one-third in the upper complex oligosaccharide form. Interestingly, SLC26A9 was almost exclusively in the complex form when expressed in MDCK cells. As in HEK cells, SLC26A4 expressed in MDCK cells ran a single major band that was sensitive to endoglycosidase H. SLC26A5 and A7 could be detected after 3 wk in selection media. Like SLC26A9, they were both expressed in mainly the complex form. Thus the processing of SLC26A5, A7, and A9 was more efficient in MDCK cells than in HEK cells. The expression levels of the other members (A2, A3, and A8) of the SLC26 family were too low to detect reliably on immunoblots even after the cells were grown 3 wk in culture.

Identification of the sites of N-glycosylation. To confirm the enzymatic deglycosylation results and identify the sites of N-glycosylation, site-directed mutagenesis experiments were performed to remove potential N-glycosylation sites within the membrane domain of the SLC26 family members. Figure 1 shows a sequence alignment and the location of potential N-glycosylation sites of all 10 human members of the SLC26 gene family. We used a putative 12 TM model with the use of this topology, facing the cytosol as a 12 TM model is supported by topology studies of BicA (58). With the use of this topology, N-glycosylation acceptor sites (N-X-S/T) were typically located between TM segments 3 and 4 in the second or in the case of SLC26A11 in the fourth, putative EC loop in the SLC26 proteins (Table 1).

To determine which N-glycosylation sites are utilized in the SLC26 family, the acceptor Asn residues were mutated individually and in combination to Asp, the residue left in place
Table 2. Cell surface expression of the human SLC26 family of anion transporter membrane proteins

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Relative Expression Levels $\pm$ SE (%)</th>
<th>Complex Oligosaccharide $\pm$ SE (%)</th>
<th>Colocalization with WGA $^{3}$ (Pearson’s r)</th>
<th>Colocalization with Calnexin $^{3}$ (Pearson’s r)</th>
<th>Cell Surface Biotinylation $^{4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC26A1</td>
<td>37.7 ± 12.4</td>
<td>34.7 ± 4.1</td>
<td>Wt 0.17 ± 0.08</td>
<td>Wt 0.76 ± 0.10</td>
<td>Wt 0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A2</td>
<td>30.7 ± 20.9</td>
<td>82.5 ± 6.6</td>
<td>Wt 0.78 ± 0.02</td>
<td>Wt 0.18 ± 0.05</td>
<td>Wt 0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A3</td>
<td>17.3 ± 8.8</td>
<td>61.2 ± 3.2</td>
<td>Wt 0.55 ± 0.07</td>
<td>Wt 0.74 ± 0.09</td>
<td>Wt 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A4</td>
<td>11.3 ± 3.7</td>
<td>0</td>
<td>Wt 0.14 ± 0.07</td>
<td>Wt 0.85 ± 0.05</td>
<td>Wt 0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A5</td>
<td>19.3 ± 8.7</td>
<td>68.5 ± 5.9</td>
<td>Wt 0.53 ± 0.04</td>
<td>Wt 0.64 ± 0.04</td>
<td>Wt 0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A6</td>
<td>100</td>
<td>54.7 ± 3.8</td>
<td>Wt 0.60 ± 0.05</td>
<td>Wt 0.69 ± 0.02</td>
<td>Wt 0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A7</td>
<td>20.8 ± 7.9</td>
<td>66.8 ± 6.4</td>
<td>Wt 0.58 ± 0.04</td>
<td>Wt 0.64 ± 0.08</td>
<td>Wt 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A8</td>
<td>2.6 ± 1.5</td>
<td>0</td>
<td>Wt 0.21 ± 0.04</td>
<td>Wt 0.70 ± 0.05</td>
<td>Wt 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>SLC26A9</td>
<td>26.9 ± 11.6</td>
<td>43.4 ± 12.7</td>
<td>Wt 0.31 ± 0.07</td>
<td>Wt 0.68 ± 0.07</td>
<td>Wt 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLC26A11</td>
<td>65.4 ± 12.5</td>
<td>37.0 ± 6.2</td>
<td>Wt 0.18 ± 0.03</td>
<td>Wt 0.69 ± 0.05</td>
<td>Wt 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Values are means ± SE. $^{1}$%Expression relative to SLC26A6 from 3 separate transfections. $^{2}$%Complex oligosaccharide determined from scans of untreated and endoglycosidase H-treated samples from 3 separate transfections (%complex: ++: >67%, ++: 33–66%, +: <33%, ±: <10% complex). $^{3}$Pearson’s coefficient of colocalization of SLC26 proteins with cell surface wheat germ agglutinin (WGA) and endoplasmic reticulum calnexin by immunofluorescence (r values: ++: >0.67, ++: 0.33–0.66, +: 0.33–0.10, ±: <0.1). $^{4}$Ratio (×10) of biotinylated bands (B) to total expression (T) (ratio: ++: >0.67, ++: 0.33–0.66, +: 0.33–0.10, ±: <0.1). *Wild-type (Wt) SLC26A8 is not N-glycosylated; N0 refers to the protein with potential N-glycosylation sites mutated.

after N-glycanase F treatment. Mutation of an N-glycosylation site is expected to produce a protein with higher mobility than the WT protein, seen as a lower apparent molecular weight by SDS gel electrophoresis. Figure 5 shows a panel of immunoblots of the intact SLC26 proteins and the various N-glycosylation mutants. The majority of SLC26 family members contained multiple N-glycosylation sites in the second EC loop (Table 1). SLC26A1 contains two utilized sites at Asn158 and 163 that increased the mobility of the upper complex band when mutated individually and to a sharp lower band when both sites were mutated. SLC26A2 has an interesting N-glycosylation pattern. This protein contains three potential N-glycosylation sites at Asn 199 and 205 in EC loop 2 and a third site at Asn357 in EC loop 4. Mutation of any of these sites alone does not result in a shift in mobility. Mutation of any two of the sites results in a shift and mutation of all three (N0) results in a further shift to a sharp lower nonglycosylated band. We conclude that there are two sites in EC loop 2 (Asn199 and 205) that are normally N-glycosylated and a third acceptor site (Asn357) in EC loop 4 with no oligosaccharide or perhaps a small high-mannose oligosaccharide. Mutation of either or both of the first two sites results in N-glycosylation, and its processing to complex, of a “cryptic” site at Asn357 that is located very close to the distal end of TM7 in EC loop 4. The N-glycosylation of Asn357 in EC loop 4, defines this region as EC.

SLC26A3 contains four potential N-glycosylation sites (Asn153, 161, 164 and 165) in EC loop 2. Mutation of all four sites (N0) resulted in a sharp nonglycosylated band (Fig. 5). Mutation of individual sites resulted in a visible shift for the N153D mutant only (Fig. 5, left) showing that these sites can each be utilized. Triple mutants containing only single acceptors sites (Fig. 5, A3, left side, middle) migrated as two bands lower in size than WT and higher than the nonglycosylated N0 mutant, indicating that all four individual sites can be utilized. Double mutants with various combination of sites mutated

AJP-Cell Physiol  •  doi:10.1152/ajpcell.00030.2014  •  www.ajpcell.org
confirmed that SLC26A3 contains multiple N-glycosylation sites in EC loop 2. The N153D/N161D mutant (Fig. 5, A3, right side, middle) was shifted down relative to the single mutants indicated that both sites were utilized. Similarly, like the N153D/N161D double mutant, the N153D/N164D and N153D/N163D double mutants were shifted also down relative to the single N153D mutant (Fig. 5, A3 right) indicating that sites Asn161, 164, and 165 were utilized in the context of the N153D mutant. Finally, the N164D/N165D double mutant was shifted down relative to the single mutants (Fig. 5, A3 far side, right) indicating both sites could be utilized. Previous work (20) identified N-glycosylation sites at Asn153, 161, and 165 in human SLC26A3 with the suggestion that nonglycosylated sites like Asn164 can be glycosylated when another single site is mutated. A similar study concluded that two of the three closely spaced sites in SLC26A3 are utilized (14) in agreement with our mutagenesis results.

SLC26A4 can utilize two sites Asn167 and 172 but not site Asn241 in the next EC loop likely because it is too close to the end of TM5 to be accessible to the oligosaccharyl transferase to be N-glycosylated. Note that SLC26A4 only contains high-mannose oligosaccharide and lectin shift assay was used to resolve the N-glycosylated (upper) and nonglycosylated (lower) proteins that normally migrated closely on SDS gels, with the nonglycosylated protein migrating at 75 kDa in this gel. The presence of a predominant nonglycosylated band indicates that SLC26A4 is not efficiently N-glycosylated in HEK cells. The WT SLC26A4 resolved as a major nonglycosylated lower band and multiple upper bands that likely represent proteins with different numbers of utilized N-glycosylation sites. Mutation of both Asn167 and 172 (and N0 in combination with Asn241) resulted in a nonglycosylated protein. Mutation of either Asn167 or 172 produced a single upper band, while the Asn241 mutant was similar to A4.WT. We surmise that SLC26A4 contains two N-glycosylation sites, either of which can be used to produce a mono-N-glycosylated protein or both sites to produce a di-N-glycosylated protein. A previous study (14) of rat prestin concluded that the protein expressed in Xenopus oocytes contained two closely spaced N-glycosylation sites at Asn163 and 166. The rat prestin ran as a single sharp band; however, its sensitivity to endoglycosidase H was not tested.

SLC26A5 utilizes two sites, Asn163 and 172 in EC loop 2, and the yield of nonglycosylated protein was decreased relative to the WT or single mutants. SLC26A6 utilizes Asn167 and 172 in EC loop 2; however, mutation of both sites resulted in a lower band that was broader than typically seen with other SLC26 members. This apparent heterogeneity is not due to N-glycosylation but may be due to incomplete unfolding of this membrane protein in SDS. SLC26A7 utilizes Asn125 and 131 in EC loop 2. Mutation of sites at Asn192, 277, or 384 in SLC26A8 did not shift the mobility of the protein, confirming the nonglycosylated status of this family member. SLC26A9 contains two utilized sites at Asn153 and 156 in EC loop 2, while SLC26A11 was unique and contained a single utilized site at Asn294 in EC loop 4.

Fig. 3. Enzymatic deglycosylation of human SLC26 family members. Panels 1–10, immunoblots of SLC26A1–9 and A11 expressed in HEK cells, respectively. C, control; H, endoglycosidase H treated (Endo H); F, N-glycanase F (PNGase F) treated. Migration positions of molecular mass (MW) markers are indicated at left and SLC26 family members at right. Cell extracts as described in Fig. 2 were incubated with no enzyme (C), 50 units of either Endo H (H) or PNGase F (F) at 37°C for 1 h. FLAG-tagged SLC26 proteins was detected by immunoblotting using a mouse anti-FLAG M2 antibody.

AJP-Cell Physiol • doi:10.1152/ajpcell.00030.2014 • www.ajpcell.org
Immunolocalization of human SLC26 family members in transfected HEK cells. The location of the FLAG-tagged constructs in transfected HEK-293 cells was determined by immunofluorescence staining and confocal microscopy. No immunofluorescence was detected using intact fixed cells but could be detected in permeabilized cells (data not shown). This shows that the NH₂-terminal FLAG epitope was not accessible to the cell exterior. The inability to detect any of the SLC family members in intact cells may be due to lack of expression at the cell surface (but see below) or that the NH₂ termini of the SLC26 proteins face the cytosol. No immunodetection of the transfected proteins obtained from Origene that were tagged at their COOH termini with the tandem myc-DDK epitopes was visible in intact fixed cells. The COOH-terminal tagged SLC26 proteins were detected after permeabilization (data not shown) showing that the COOH termini of the SLC26 proteins located at the cell surface face the cytosol as predicted for location of the STAS domain.

SLC proteins were readily detected by immunofluorescence in transfected cells that were fixed and permeabilized. All SLC26 family members colocalized with the WGA cell surface marker (Fig. 6). There was little intracellular staining of SLC26A2, which showed a predominant cell surface staining. The other SLC26 proteins showed intracellular staining colocalizing with the ER marker calnexin (Fig. 7). This is consistent with the detection of high-mannose forms of these proteins on immunoblots.

Table 2 lists the Pearson’s coefficient ($r$) for colocalization for the SLC26 proteins with the cell surface marker WGA and calnexin. As expected, SLC26A2 had the highest coefficient (0.78) with the majority of the immunolocalized protein overlapping with WGA at the cell surface and the lowest overlap with calnexin. SLC26A3, A5, A6, and A7 had coefficients of between 0.33 and 0.66, indicating significant localization to the plasma membrane. SLC26A1, A4, A8, and A9 had Pearson’s coefficient <0.33, indicating less overlap with WGA and a lower level of plasma membrane localization. There was a strong correlation of the Pearson’s coefficient with the percent complex oligosaccharide (Table 1), indicating the level of processing to complex oligosaccharide reflected trafficking to the cell surface. Note, however, that SLC26A4, which contained only high-mannose oligosaccharide, did colocalize weakly ($r = 0.14$) at the cell surface with WGA by immunofluorescence. Furthermore, SLC26A8 that contained no N-linked oligosaccharide was also weakly ($r = 0.21$) expressed at the cell surface. Therefore, some fraction of SLC26A4 and A8 although they do not contain complex oligosaccharide can traffic to the cell surface.

Figures 5 and 6 also show immunofluorescence images of cells expressing the nonglycosylated mutants. The staining patterns of the nonglycosylated mutants were similar to the WT glycosylated proteins. Table 2 lists the Pearson’s coefficients for the nonglycosylated versions of the SLC26 family. The values were similar to the WT proteins, indicating that N-glycosylation was not essential for cell surface expression of the SLC26 proteins in HEK cells. All of the mutants showed intracellular staining colocalizing with the ER marker calnexin; however, all could be detected at the cell surface. Thus N-glycosylation of SLC26 proteins is not a strict requirement for their exit from the ER and trafficking to the plasma membrane. This is particularly noteworthy in the case of SLC26A4, which
is present in solely high-mannose form and SLC26A8, which does not contain any N-linked oligosaccharide. Table 2 also provides the Pearson’s correlation values for the nonglycosylated (N0) versions of the SLC family. Most of the values were similar to, or slightly lower, than the values for the WT proteins. Thus N-glycosylation does not play an essential role in the trafficking of the SLC26 family of proteins to the cell surface but may facilitate cell surface expression in some cases, perhaps by stabilizing the protein at the level of the plasma membrane.

Cell surface biotinylation. The cell surface expression of SLC26 family members was confirmed using a cell-surface biotinylation assay. All family members could be biotinylated to varying extents, while an internal marker, actin was not

Fig. 5. Identification of N-glycosylation sites in human SLC26 family members by site-directed mutagenesis. Panels 1–10: immunoblots of SLC26A1-9 and A11 expressed in HEK cells, respectively. Migration positions of molecular mass (MW x 10^3) markers are indicated at left and SLC26 family members and endogenous GAPDH at right. Asparagine residues in putative acceptor sites (-NXS/T-) were mutated individually or in combination to aspartate residues as indicated. NxyzD, residue number of mutated asparagine residue; N0, all acceptor sites mutated producing nonglycosylated protein. Proteins in cell extracts were resolved by 8% SDS-PAGE, except SLC26A4 protein where a lectin gel shift assay was used to better separate the high-mannose (upper) from nonglycosylated band (lower). FLAG-tagged SLC26 proteins were detected by immunoblotting using a mouse anti-FLAG M2 antibody.
Fig. 6. Immunolocalization of wild-type (WT) and nonglycosylated (N0) human SLC26 family members SLC26A1-9 and A11 in transfected HEK cells prestained with wheat germ agglutinin (WGA). Glycoproteins displaying complex oligosaccharides on the cell surface of intact HEK cells were labeled with WGA-Alexa488 (green) at 37°C for 10 min. The cells were washed, fixed and permeabilized and then stained with mouse anti-FLAG M2 followed by anti-mouse IgG-cy3 (red) for total SLC26 protein. Cells were examined using a LSM510 confocal fluorescence microscope. Yellow represents colocalization of SLC26 protein at the plasma membrane with the cell surface marker WGA.
Fig. 7. Immunolocalization of WT and nonglycosylated human SLC26 family members SLC26A1-9 and A11 in transfected HEK cells with calnexin, an endoplasmic reticulum (ER) marker. WT and nonglycosylation mutant (N0) of SLC26A1-9 and A11 with an NH2-terminal FLAG tag were transfected into HEK cells with Lipofectamine LTX and Plus and grown for 24 h on glass cover slips. Cells were fixed and permeabilized. The total expression of SLC26 proteins were detected using a mouse anti-FLAG M2 antibody, and calnexin was then detected using a rabbit anti-calnexin antibody followed by secondary antibody anti-mouse IgG-Alexa488 (green) and anti-rabbit Cy3 (red) antibody. Cells were examined using a LSM510 confocal fluorescence microscope. Yellow represents colocalization of SLC26 proteins with the ER marker calnexin, and Pearson coefficients (r values) were calculate to estimate their overlap.
biotinylated. Figure 8 shows an immunoblot of total (T) SLC26 proteins, the unbound (U) fraction not captured by streptavidin beads, and the 10-fold concentrated sample of bound (B) protein eluted from the beads with SDS sample buffer. Both the complex and high-mannose bands of SLC26A1 could be biotinylated showing that not only was the complex form expressed at the cell surface but also the high-mannose form. The complex band of SLC26A1 was enriched in the bound fraction relative to the total indicating preferential expression at the cell surface. Note that actin was not labeled in this experiment, showing that the biotinylation reagent had not penetrated the cell. The complex form of SLC26A2 could be readily biotinylated consistent with its predominant cell surface expression. SLC26A3 showed a predominant biotinylation of the upper complex band with little labeling of the lower high-mannose band, as did SLC26A5, A6, and A7. This shows that the complex form had trafficked to the cell surface, while the high-mannose form was largely intracellular localized to the ER as indicated by the immunofluorescence colocalization with calnexin. The high-mannose band of SLC26A4 could be weakly biotinylated, showing that it could traffic to the cell surface without processing. We have made a similar observation with the human anion exchanger 1 (AE1, band 3) expressed in transfected HEK cells (29). SLC26A9 and A11 were weakly biotinylated. In addition, no biotinylated bands were seen for SLC26A8, although its total expression level was very low, too low perhaps to detect minor amounts of biotinylated protein.

The amount of biotinylated protein recovered was very low and did not provide a quantitative measure of cell surface expression. This may be due to a paucity of reactive lysine residues on the cell surface or differences in the size of accessible extracellular loops. However, the ratio (B/T) of biotinylated protein in the bound fraction (B) to total protein (T) provided an estimate of the different abilities of the proteins to be biotinylated (Table 2). Proteins showing the highest level of cell surface biotinylation include SLC26A1, A2, and A5, with SLC26A3, A4, and A6 showing intermediate
levels and SLC26A7-A11 showing very low levels. The nonglycosylated proteins typically showed a lower level of biotinylation. The nonglycosylated versions of SLC26 family members that showed the highest or intermediate levels of biotinylation (SLC26A1, A2, A3, A5, and A6) could still be detected at the cell surface by biotinylation. Thus the biotinylation results support the conclusion that N-glycosylation is not a requirement for cell surface expression of the SLC26 family members. However, the level of biotinylation was lower in most cases showing some decrease in cell surface biotinylation for the nonglycosylated proteins (Table 1). This indicates that N-glycosylation may stabilize the SLC26 proteins at the level of the plasma membrane.

**DISCUSSION**

In this article we show that all members of the SLC26 family of anion transport protein, with the exception of SLC26A8, are N-glycosylated at specific sites in the second, or in the case of SLC26A11, the fourth EC loop. This establishes the topology of these two regions of the protein that is consistent with the topology models summarized in Fig. 9. Although the amino acid sequences of EC loop 2 are poorly conserved, most family members contain N-glycosylation acceptor sites (-NXS/T-) in this region (Fig. 1). A survey of membrane glycoproteins showed that they were typically N-glycosylated on a single loop close to the NH2-terminal region of the protein and the SLC26 family shows these features (27). The majority of SLC26 family members (A1, A2, A3, A4, A5, A6, A7, and A9) contain multiple N-glycosylation sites in EC loop 2 (Table 1). Interestingly, SLC26A11 did not contain an acceptor site in the second EC loop, which is shorter than the other SLC26 family members but rather contains a single site at position Asn294 in an expanded EC loop 4 (Fig. 1). This finding shows that this region, which is a short turn in most SLC26 family members, is EC. Mutation of the single N-glycosylation site in EC loop 2 of SLC26A8 did not alter the mobility of the protein, consistent with the observation that A8 is not N-glycosylated. The site in SLC26A8 at Asn192 is located very close to the next TM segment; too close to be efficiently N-glycosylated by the ER oligosaccharyl transferase (27). SLC26A2 is normally N-glycosylated at two sites in EC loop 2 but contains a cryptic site in EC loop 4 that is N-glycosylated if one or both of the endogenous sites are mutated. N-glycosylation mutagenesis has identified two EC regions, EC loops 2 and 4 in the human SLC26 family of anion transporters, separated by four TM segments.

Table 1 summarizes the location of the utilized sites, and Fig. 9 shows their location in folding models of all 10 SLC26 family members. SLC26A1 contains two sites of N-glycosylation at Asn153 and 161 in the second EC loop. SLC26A2 contains two sites of N-glycosylation at Asn199 and 205 in EC loop 2. Mutations of one or both of these sites results in the utilization of a cryptic site in EC loop 4. Both SLC26A1 and A2 contain a pair of closely spaced conserved cysteine residues in EC loop 2 just distal to the N-glycosylation sites (Fig. 1), which may form tight turn linked by a disulfide bond. Mutation of the cysteine residues in SLC26A1 and A2 did not affect the level or pattern of expression of these proteins when expressed in HEK cells (data not shown). SLC26A2, an apical protein, traffics very efficiently to the cell surface in transfected HEK-293 cells. SLC26A3 is N-glycosylated at four sites (Asn153, 161, 164, and 165) in the second EC loop. In agreement with previous studies in transfected Cos-1 cells (7) and HEK cells (20), human SLC26A3 ran as two bands on SDS gels, an upper complex form and a lower high-mannose form. The oligosaccharide on SLC26A3 has been reported (20) to provide some protection against proteolysis and to be important for its cell surface expression in CHO and MDCK cells but not for transport activity per se.

SLC26A4 contains two N-glycosylation sites at Asn167 and 172 in EC loop 2 that are not processed to complex oligosaccharide in HEK cells. Loss of N-glycosylation sites on SLC26A4 has been reported not to alter its cell surface expression or targeting to the apical membrane but did change the sensitivity of its transport activity to extracellular pH (3). Expression of the mouse protein in HEK cells resulted in the production of a high-mannose form of the protein with small amount of the complex form (3). Our studies showed that the human protein was exclusively in the high-mannose form. Yoon et al. (69) found both complex and high-mannose forms of the human protein in transfected HEK cells. SLC26A5, SLC26A6, SLC26A7, and SLC26A9 also each contain two N-glycosylation sites in the second EC loop at Asn163 and 166, Asn125 and 131, Asn167 and 172, and Asn153 and 156, respectively. Unlike SLC26A4, these sites are processed to complex oligosaccharide.

SLC26A8 and A11 have unique N-glycosylation patterns. A8 is not N-glycosylated even though it contains consensus sites, while A11 is N-glycosylated at a single site (Asn294) in the fourth EC loop rather than the second EC loop as is commonly found for most other family members.

The SLC26 proteins, with the possible exception of SLC26A8, can traffic to the plasma membrane in transfected HEK cells; however, a significant fraction of the proteins were localized intracellularly in the ER. This is likely due to overexpression, which overwhelms the secretory pathway. SLC26A2, however, is both well expressed and very efficiently converted to the complex form where it is present prominently at the cell surface.

Table 2 provides a summary of the cell surface expression of the SLC26 proteins and the effect of mutating the N-glycosylation sites. SLC26A2 shows the highest level of cell surface expression, and removing the N-glycosylation had little effect on its cell surface expression. Biotinylation assays showed that removing the N-glycosylation sites reduced but did not prevent the cell surface expression of SLC26A1, A3, A5, and A6. SLC26A4 contained only high-mannose oligosaccharide but could traffic to the cell surface and mutating these sites decreased its cell surface expression. SLC26A7 and A9 were weakly biotinylated, which is consistent with a low level of complex oligosaccharide. In addition, removing their N-glycosylation sites diminished cell surface expression. SLC26A8 is nonglycosylated, poorly expressed in HEK cells, and poorly expressed at the cell surface.

Localization of the N-terminal FLAG tag and the COOH-terminal myc-DDK tag to the cytosolic side of the plasma membrane shows that the SLC26 proteins span the membrane an even number of times, with both termini in the cyanobacterial member of the SulP/SLC26 family, which was shown to contain 12 TM segments with both termini in the
Fig. 9. Topology models of the 10 human SLC26 family of anion transporters showing the cytosolic location of the NH₂ and COOH termini and the position of the EC loops determined by the utilized N-glycosylation sites shown as green trees. The cryptic N-glycosylation site in SLC26A2 in colored in orange. Nonglycosylated sites are in red, and the consensus sites in the cytosolic N terminus and COOH-terminal STAS domain that were not examined are shown in blue.
cytosolic localization of the NH2 and COOH termini and EC
their stability at the level of the plasma membrane. The
prevent cell surface expression of the proteins but may affect
the cell surface. Removal of the
unique in not being
cells. SLC26A4 retained a high-mannose structure and was the
with its ability to traffic efficiently to the cell surface in HEK
efficiently processed to complex oligosaccharide consistent
N-glycosylation sites were localized on two EC loops. Most SLC26 family members are N-glycosylated at multiple sites on the second EC loop, while SLCAs1 is N-glycosylated at a single site on EC loop 4 and SLC26A2 contains a cryptic site in EC loop 4. The efficiency of processing of the N-linked glycan varied, with SLC26A2 being very efficiently processed to complex oligosaccharide consistent with its ability to traffic efficiently to the cell surface in HEK cells. SLC26A4 retained a high-mannose structure and was the most highly localized family member to the ER. SLC26A8 was unique in not being N-glycosylated, and it trafficked poorly to the cell surface. Removal of the N-glycosylation sites did not prevent cell surface expression of the proteins but may affect their stability at the level of the plasma membrane. The cytosolic localization of the NH2 and COOH termini and EC N-glycosylation sites allowed the construction of a more detailed topology models for the SLC26 family of anion transporters that contain an even number of TM segments with the NH2 and COOH termini facing the cytosol.

ACKNOWLEDGMENTS

Dr. Michael Romero (Mayo) is thanked for providing cDNA for human SLC26A2, 3, 6, and 9 in pGEMH and Dr. Gérard Gacon (INSERM U1016/ CNRS UMR8104, Paris) is thanked for providing the cDNA for human (TAXI) SLC26A8 in pCDNA3. Mengyang Xu is thanked for assistance with some of the experiments. Chloé Rapp is thanked for editing the manuscript.

GRANTS

This work was supported by an Operating Grant (MOP120493) from Canadian Institutes of Health Research.

DISCLOSURES

No conflicts of authorship or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.L. and F.X. performed experiments; J.L., F.X., and R.R. interpreted results of experiments; J.L. prepared figures; J.L., F.X., and R.R. edited and revised manuscript; R.R. conception and design of research; R.R. drafted manuscript; R.R. approved final version of manuscript.

REFERENCES

13. Detro-Dassen S, Schanzler M, Lauks H, Martin I, zu Berstenhorst S, Heneghan JF, Akhavein A, Salas MJ, Shmukler BE, Karniski LP. Homologous mutations in SLC26A3, A5, and A6 are cytosolic. It is possible that the presence of these tags could influence the folding, expression and localization of the proteins; however, the NH2-terminal regions of the SLC26 family are poorly conserved and not known to be involved in interactions in contrast with the case for the COOH-terminal STAS domain. Many SLC26 family members end with a cytosolic PDZ binding motif, permitting the interaction of PDZ binding proteins that can form cytosolic bridges to other proteins such as CFTR (1).

In this study we have shown that the human SLC26 family members can traffic to the cell surface with varying ability in transfected HEK cells and that they span the membrane an even number of times with the NH2 and COOH termini facing the cytosol. N-glycosylation sites were localized on two EC loops. Most SLC26 family members are N-glycosylated at multiple sites on the second EC loop, while SLC26A1 is N-glycosylated at a single site on EC loop 4 and SLC26A2 contains a cryptic site in EC loop 4. The efficiency of processing of the N-linked glycan varied, with SLC26A2 being very efficiently processed to complex oligosaccharide consistent with its ability to traffic efficiently to the cell surface in HEK cells. SLC26A4 retained a high-mannose structure and was the most highly localized family member to the ER. SLC26A8 was unique in not being N-glycosylated, and it trafficked poorly to the cell surface. Removal of the N-glycosylation sites did not prevent cell surface expression of the proteins but may affect their stability at the level of the plasma membrane. The cytosolic localization of the NH2 and COOH termini and EC N-glycosylation sites allowed the construction of a more detailed topology models for the SLC26 family of anion transporters that contain an even number of TM segments with the identification of EC loops 2 and 4 shown in Fig. 9.


