Quail Sulf1 Function Requires Asparagine-linked Glycosylation

Received for publication, August 14, 2007 Published, JBC Papers in Press, September 12, 2007, DOI 10.1074/jbc.M706744200

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The heparan sulfate endosulfatasases Sulf1 and Sulf2 are cell-surface enzymes that control growth factor signaling through regulation of the 6-O-sulfation states of cell-surface and matrix heparan sulfate proteoglycans. Here, we report that quail Sulf1 (QSulf1) is an asparagine-linked glycosylated protein. Domain mapping studies in combination with a protein glycosylation prediction program identified multiple asparagine-linked glycosylation sites in the enzymatic and C-terminal domains. Glycosylation inhibitor studies revealed that glycosylation of QSulf1 is essential for its enzymatic activity, membrane targeting, and secretion. Furthermore, N-glycanase cleavage of asparagine-linked sites in native QSulf1 provided direct evidence that these N-linked glycosylation sites are specifically required for QSulf1 heparin binding and its 6-O-desulfation activity, revealing that N-linked glycosylation has a key role in the control of sulfatase enzymatic function.

Sulfatases (Suls) are cell-surface heparan sulfate (HS) endosulfatasases that regulate the 6-O-sulfation of HS chains on cell-surface and matrix proteoglycans (1). The 6-O-sulfation states of HS proteoglycans differentially affect extracellular signaling during embryogenesis, tissue regeneration, and cancer progression (2). In avian embryos, antisense inhibition of Sulf1 blocks Wnt-dependent myoD expression in myogenic progenitors (2). In addition, Sulf deficiency leads to a disruption of glial cell line-derived neurotrophic factor-dependent esphaghal innervation, leading to postnatal death and severe growth defect in Sulf double mutant mice (3). Sulfs not only play essential roles in embryos, but also regulate tumor growth. For example, primary breast cancer cells down-regulate Sulf expression to enhance growth, survival, and angiogenesis in response to HS-dependent growth factor signaling (4–6), whereas pancreatic tumors increase Sulf expression to promote Wnt signaling required for pancreatic tumor progression (7).

Both transcriptional and post-translational mechanisms control Sulf expression and its important roles in extracellular signaling. A number of previous studies have characterized dynamic mRNA expression of Sulf1 and Sulf2 in embryonic and adult tissues (4, 8, 9). Sulfs also undergo post-translational modification, including cleavage by furin-like proteases and N-glycosylation (10). Whether these post-translational modifications regulate the enzymatic properties of Sulfs is unknown. In this study, we investigate the functional roles of Sulf N-linked glycosylation.

Glycosylation of membrane and secreted proteins involves a series of enzymatic reactions mediated by the activities of oligo- and monosaccharide transferases. N-Linked protein glycosylation occurs post-translationally and is initiated in the lumen of the endoplasmic reticulum (ER), concomitant with translocation, by the transfer of a core N-linked glycosylation unit onto acceptor asparagine residues in the tripeptide sequence AsnXaa-(Ser/Thr) (11). This first step is catalyzed by oligosaccharyltransferase, a component of the protein translocation machinery in the ER membrane. As the newly glycosylated protein is transported from the ER to the Golgi apparatus, trimming and addition of other sugar residues occur (12). In addition to asparagine-linked glycosylated chains, which can be cleaved by N-glycanase, there are other types of glycosylation, including serine/threonine (O-linked) and sialic acid-linked sugar chains (12–14), which can be cleaved using O-glycanase and sialidase A, respectively. N-Linked glycosylation is often essential for proper protein folding, stability, intracellular transport, secretion, and functional activity (15, 16). In this study, we show that both avian and human Sulfs are glycosylated at multiple N-linked sites and that this glycosylation is required for quail Sulf1 (QSulf1) substrate binding, enzymatic activity, membrane targeting, and secretion.

MATERIALS AND METHODS

Plasmids—QSulf1 and mutant QSulf1 with a deleted hydrophilic domain (QSulf1-ΔHHD) were described previously (3). All QSulf1 clones were tagged with Myc-His at the C terminus. QSulf1 with a deleted enzymatic domain (QSulf1-ΔN) was generated by site-directed mutagenesis using the following primers: 5’-GATGACAAAGATGGAAGCTTGAGCTGTCAG-GGGAAAG-3’ (forward) and 5’-CTTCCCTTTGACCATGTC-ACGTCCTACATTTCCATCTC-3’ (reverse). Human Sulf1 and Sulf2 were provided by Dr. Steven D. Rosen (University of California, San Francisco).

Cell Culture—Human embryonic kidney (HEK) 293T cell lines were cultured in Dulbecco’s modified Eagle’s medium (Mediatech) with 10% fetal bovine serum (HyClone) and antibiotics (Cellgro). Cells were stably transfected with QSulf1 as described previously (1). Stably transfected cells were incubated for up to 72 h with tunicamycin (1 μg/ml; Alexis Bio-
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**FIGURE 1.** QSulf1 is a glycosylated protein. A, SDS gel electrophoresis and Western blot (WB) analysis of the molecular mass of Myc/His-tagged QSulf1 expressed in stably transfected HEK293 T cells treated with tunicamycin for 72 h. Blots were probed with anti-Myc (upper panel) and anti-His (lower panel) antibodies to detect QSulf1 expression. B, SDS gel electrophoresis and Western blot analysis of time-dependent changes in the molecular mass of QSulf1 following tunicamycin treatment of HEK293 cells expressing Myc/His-tagged QSulf1 and control cells transfected with pcDNA3.1. Gel loading was monitored by actin expression using anti-actin antibody. C, analysis of the molecular mass of Myc/His-tagged human Sulf1 (HSulf1) and Sulf2 (HSulf2) expressed in transiently transfected HEK293 cells treated with tunicamycin for 24 h. Blots were probed with anti-Myc antibody to detect human Sulf1 and Sulf2 expression. Red and blue arrowheads mark the mobilities of the 100-kDa unglycosylated and 125-kDa glycosylated proteins, respectively. The protein molecular masses are indicated in kilodaltons.

**FIGURE 2.** QSulf1 is an N-linked glycosylated protein. A, SDS gel electrophoresis and Western blot (WB) analysis of Myc/His-tagged QSulf1 isolated from stably transfected HEK293 cells after digestion of cell extracts with specific glycosidases as shown. QSulf1 was detected with anti-Myc antibody. pcDNA3.1-transfected cells served as a negative control. Gel loading was monitored by actin expression using anti-actin antibody. Extracts from tunicamycin-treated cells served as molecular mass markers for deglycosylated QSulf1. The protein molecular masses are indicated in kilodaltons. B, SDS gel electrophoresis and Coomassie Blue staining of partially purified QSulf1 (left panel) and Western blot analysis of Myc/His-tagged QSulf1 isolated from stably transfected HEK293T cells without and with tunicamycin treatment following N-glycanase digestion by denaturing methods (right panel).

Incubation buffer, and 1 μl of N-glycanase, followed by incubation at 37 °C for different time points (0–5 days).

**Western Blot Assays**—Western blot assays were conducted as described previously (2). The primary antibodies were purchased from Santa Cruz Biotechnology, Inc.: mouse anti-Myc (9E10, 1:2000), rabbit anti-His (1:2000), and rabbit anti-actin (1:2000). Peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories: goat anti-mouse (1:4000) and goat anti-rabbit (1:4000).

**Protein Purification**—Protein purification was performed using nickel-nitrilotriacetic acid resins. QSulf1-expressing 293T cells were lysed in buffer containing 0.1% Triton X-100, 50 mM Tris (pH 8.0), 500 mM NaCl, and protease inhibitors. Cell lysate was incubated with nickel-nitrilotriacetic acid beads to allow binding. After washing, proteins bound to the resin were eluted with an imidazole gradient (0.5–1 M).

**Cell Fractionation**—Subcellular fractionation was performed using the FractionPREP cell fractionation system kit (BioVision, Inc.). Briefly, cells were lysed in cytosol extraction medium. After centrifugation, the supernatant was collected as the cytosolic fraction. The pellet was suspended in membrane extraction buffer to separate the membrane/particulate fraction in the supernatant. The pellet was further suspended in...
nuclear extraction buffer, and after centrifugation, the super-
natant was collected as the nuclear fraction, whereas the pellet
was boiled in Laemmli buffer as the cytoskeletal fraction of the
cell.

Heparin Binding Assays—Protein extracts (50 μg) isolated
from the Triton-soluble fraction were incubated with 7 μl of
heparin-conjugated beads in phosphate-buffered saline (PBS; a
total of 50 μl) for 2 h at either 37 or 4 °C. After binding, samples
were centrifuged at 13,000 rpm to separate the beads from
unbound protein. Beads were washed with PBS plus protease
inhibitors, and protein bound to heparin beads was analyzed by
SDS gel electrophoresis and Western blotting.

Immunocytochemistry—Cells were cultured on acid-cleaned
coverslips. To assay cell-surface QSulf1 and HS, unfixed live
cells were incubated with Cy3-conjugated anti-Myc antibody
9E10 (1:100; Sigma) for 1 h or with fluorescein isothiocyanate
(FITC)-conjugated wheat germ lectin (1:200; Sigma) for 20 min
at 37 °C. To assay intracellular protein expression, cells were fixed
with 4% parafomaldehyde in PBS for 15 min, followed by permeabi-
lization with 0.01% Triton X-100 in PBS. After permeabilization, non-
specific staining was blocked by incubating cells in PBS containing
3% bovine serum albumin, followed by incubation with the antibodies.
After washing with PBS, the antigen-antibody complex was visual-
ized by fluorescence microscopy. The primary antibodies used were
rabbit anti-ER marker calreticulin (1:1000; Abcam), rabbit anti-mouse
Sulf1 (1:50), chicken anti-syn-de-
nan-4 (a gift from Dr. D. D. Cornel-
lisson), and rabbit anti-c-Met (Santa
Cruz Biotechnology, Inc.).

QSulf1 Enzyme Assays—HEK293T cell cultures transfected with QSulf1
and control plasmids were lysed in
500 μl of 0.25% Triton X-100 with
protease inhibitors. Freshly pre-
cipitated cell lysates were dialyzed
at 4 °C overnight in Tris-buffered
saline before sulfate release assays as
described previously (2). Samples
were digested for 6 h at 37 °C, fol-
lowed by assay of 35S release from
radiolabeled glycosaminoglycans by
scintillation counting.

Reverse Transcription-PCR—Re-
verse transcription-PCR was per-
formed as recommended by Invitro-
gen. Briefly, RNA was isolated
from rhabdomyosarcoma formed
in Patched+/−/− CD1 mice using
TRizol reagent, followed by DNase I
treatment and reverse transcrip-
tion. PCR was conducted using the following primers: mouse
Sulf1 (MSulf1), 5′-TGTTTGTCGCAACGGCATC-3′ (for-
ward) and 5′-GGACCACGAATGAAGAAAGGC-3′ (reverse);
and 18 S rRNA, 5′-GTAACCCGTTGAACCCCATT-3′ (forward) and 5′-CCATCCAACTGGTAGTAGGC-3′
(reverse).

Mice—Patchet heterozygous mice (17) were bred and main-
tained in accordance with Institutional Animal Care and Use
Committee guidelines. Mice were humanely killed, and tumor
tissue was dissected for the studies described below.

Primary Rhabdomyosarcoma Cell Culture—Patchet+/−/−
mouse rhabdomyosarcoma tissue was dissected, rinsed with
Hanks’ balanced salt solution, and minced prior to enzymatic
dissociation with 0.1% collagenase (Worthington) and physical
trituration. Dissociated tissues were filtered through 100- and
40-μm cell sieves. Cells were propagated in proliferation
medium (Ham’s F-10 medium (Cellgro), 20% fetal bovine

FIGURE 3. Predicted N-linked sites and domain mapping of N-linked sites of QSulf1. A, schematic domain
structures of native QSulf1 and mutants QSulf1-ΔHD and QSulf1-ΔN with the predicted N-linked glycosylation
sites (N), the cysteine residue essential for catalytic activity (C877A), and the putative furin cleavage sites (X). aa,
amino acids. B, molecular mass analysis of QSulf1, QSulf1-ΔHD, and QSulf1-ΔN isolated from transiently trans-
fected HEK293T cells after digestion with or without N-glycanase. Extracts from tunicamycin-treated QSulf1-
expressing cells provided a marker for unglycosylated QSulf1. The protein molecular masses are indicated in
kilodaltons. WB, Western blot.
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**A.**

| HEK 293 cells | WB: anti-my | Heparin Binding | Tunicamycin (1 µg/ml; 24 h): | Loading control |
|---------------|-------------|-----------------|-------------------------------|----------------|
| pcDNA3.1 plasmid | : | : | + | - |
| QSulf1 cDNA | : | : | + | - |

**B.**

![Graph](image_url)

**FIGURE 4. Glycosylation of QSulf1 is required for its heparin binding and enzymatic activity using tunicamycin treatment.** A, shown are the results from the analysis of heparin-binding and non-heparin-binding Myc/His-tagged QSulf1 proteins expressed in untreated and tunicamycin-treated (24 h) HEK293T cells. Cell lysates were incubated with heparin-conjugated beads for 2 h at 37 °C before Western blot (WB) assay of QSulf1 bound to heparin beads and in the supernatant. B, QSulf1 isolated from tunicamycin-treated HEK293T cells had reduced enzymatic activity, as shown in [35]SO4 release assays using metabolically radiolabeled glycosaminoglycan substrates. HEK293 cells transfected with pcDNA3.1 served as a negative control. The Western blots in the lower panels show that equivalent amounts of QSulf1 proteins were present in cell extracts utilized for enzyme assays. Red and blue arrowheads mark the mobilities of the 100-kDa unglycosylated and 125-kDa glycosylated QSulf1 proteins, respectively. Anti-actin antibody was used to monitor gel loading.

serum, 5 ng/ml fibroblast growth factor-2 (Promega Corp.), 2% chick embryo extract, and 1% penicillin/streptomycin) on ECL-coated tissue culture dishes (Upstate).

**RESULTS**

**QSulf1 Is an Asparagine-linked Glycosylated Protein—**NetNGlyc, a glycosylation analysis program (www.cbs.dtu.dk/services/NetNGlyc/), predicts that QSulf1 has multiple N-linked sites, but no O-linked or sialic acid-linked glycosylation sites. Consistent with this prediction, we observed a discrepancy in the molecular mass of Myc/His-tagged QSulf1 based on its amino acid sequence (100 kDa) and its mobility on SDS gels (~125 kDa) (Fig. 1A). To investigate whether N-glycosylation accounts for this molecular mass shift, HEK293T cells stably expressing Myc/His-tagged QSulf1 were treated with tunicamycin, an inhibitor of N-linked glycosylation and the formation of N-glycosidic protein-carbohydrate linkages (18, 19). Protein lysates were then subjected to molecular mass analysis by SDS gel electrophoresis and Western blotting. Tunicamycin treatment caused a progressive shift in QSulf1 molecular mass (from 125 to 100 kDa) over a period of 2–24 h (Fig. 1B), suggesting that QSulf1 has N-linked glycosylation. Treating the cells with tunicamycin for >24 h results in reduced cell anchorage (data not shown) (12). Therefore, the 24-h time point was used for all experiments, as this time was sufficient to inhibit glycosylation without disrupting cell adhesion. Tunicamycin treatment of cells expressing human orthologs of Sulf1 and Sulf2 also caused a similar shift in molecular mass on SDS gels (Fig. 1C), providing evidence that Sulf N-glycosylation is evolutionarily conserved.

To confirm that Suls are subject only to N-linked glycosylation, QSulf1 protein extracted from stably expressing HEK293T cells was treated with N-glycanase, O-glycanase, or sialidase A under denaturing conditions to allow complete deglycosylation, followed by molecular mass analysis by electrophoresis on SDS gels. N-Glycanase selectively reduced the mobility of QSulf1 to 100 kDa, thus further identifying QSulf1 as an N-glycosylated protein (Fig. 2A). Consistent with the prediction by NetNGlyc, O-glycanase or sialidase A had no effect on the molecular mass of QSulf1, establishing that QSulf1 is exclusively N-glycosylated.

QSulf1 expressed in tunicamycin-treated cells had the same reduced molecular mass as QSulf1 deglycosylated by N-glycanase (compare Figs. 1 and 2A). This result was likely due to a complete inhibition of N-linked glycosylation by tunicamycin. Alternatively, tunicamycin may cause partial degradation of QSulf1 nonspecifically. To distinguish between these two possibilities, QSulf1 expressed in untreated or tunicamycin-treated cells was digested by N-glycanase under denaturing conditions. Although N-glycanase reduced the molecular mass of untreated and partially purified QSulf1 from 125 to 100 kDa, N-glycanase did not further reduce the molecular mass of unglycosylated and non-purified QSulf1 expressed by tunicamycin-treated cells (Fig. 2B). These results establish that QSulf1 is N-glycosylated and that tunicamycin treatment inhibits QSulf1 glycosylation without affecting the stability of QSulf1.

**Predicted N-Linked Sites of QSulf1 and Its Domain Mapping—**To map the N-linked glycosylation sites of QSulf1, we scanned the Sulf sequences using the NetNGlyc program in the protein sequence context of the Asn-Xaa-(Ser/Thr) tripeptide sequon (Fig. 3A). Human and mouse Sulf1 orthologs have 10 putative N-linked glycosylation sites (10), whereas QSulf1 was predicted to have 11 sites: seven sites in the enzymatic domain located at residues 64, 111, 131, 148, 170, 197, and 240; two sites in the HD domain (residues 527 and 620); and two sites in the C-terminal domain (residues 64 and 170) (Fig. 1A). Among the seven N-linked sites in the enzymatic domain, the tripeptide sequence of the two N-linked sites at residues 64 and 170 are not conserved across vertebrate species upon performing multiple alignment of Sulf1 from different species (data not shown), suggesting that these sites may not be functional.

We hypothesized that QSulf1 has seven functional N-linked sites to fully account for the discrepancy in its predicted (100 kDa) and observed (125 kDa) molecular masses, as each
N-linked glycosylation contributes ~3.5 kDa to a protein (3.5 × 7 = 24.5 kDa). However, the NetNGlyc program predicts nine conserved N-glycosylation sites in QSulf1. To test the functionality of these sites, we generated deletion mutations of the enzymatic domain (QSulf1-ΔN) and the hydrophilic domain (QSulf1-ΔHD) (supplemental Fig. 1A) (3). Each deletion mutant protein was expressed in untreated or tunicamycin-treated HEK293T cells. QSulf1-ΔHD underwent a full 25-kDa molecular mass shift when expressed in tunicamycin-treated cells or when extracted proteins were treated with N-glycanase under denaturing condition (Figs. 2B and 3B), suggesting that the HD is unglycosylated. Furthermore, the HD alone expressed in HEK293T cells did not show a tunicamycin-induced shift in the molecular mass (supplemental Fig. 1C), thus establishing that the HD does not contain functional N-linked glycosylation sites. By contrast, QSulf1-ΔN showed a shift of ~7 kDa when N-glycosylation was blocked (Fig. 3B), indicating that two glycosylation sites are located in the C terminus. Thus, by deduction, the N-terminal enzymatic domain has five functional glycosylation sites that are likely located at conserved residues 111, 131, 148, 197, and 240.

Glycosylation of QSulf1 Is Essential for Binding of Sulfatase Heparin and Its 6-O-Endosulfatase Enzymatic Activity—N-Linked glycosylation of Sulfs is evolutionarily conserved, suggesting the functional importance of this post-translational modification for these enzymes. To investigate whether glycosylation is required for the enzymatic function of QSulf1, we compared the substrate binding and HS 6-O-endosulfatase activities of unglycosylated and glycosylated forms of QSulf1. QSulf1 was isolated from stably transfected HEK293T cells that were treated with and without tunicamycin. We assayed heparin binding to isolated QSulf1 after incubation with heparin-conjugated acrylic beads. We observed that the heparin binding to unglycosylated QSulf1 was reduced compared with that to glycosylated QSulf1 (Fig. 4A), establishing a role for glycosylation in QSulf1 binding to its heparin substrate. We also assayed the enzymatic activity of isolated QSulf1 using 35S-labeled glycosaminoglycan substrates. Compared with QSulf1 from untreated cells, which released significant 35S radioactivity, QSulf1 from tunicamycin-treated cells exhibited minimal sulfate release activity, and residual activity was likely due to low levels of glycosylated QSulf1 remaining after tunicamycin treatment (Fig. 4B). These findings suggest that glycosylation is required for QSulf1 binding to heparin substrate and its 6-O-endosulfatase activity.

The loss of substrate binding and enzymatic activity of unglycosylated QSulf1 after tunicamycin treatment may be caused nonspecifically, as tunicamycin is a general inhibitor of protein N-glycosylation. To provide further evidence that N-linked gly-
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Glycosylation targets QSulf1 to the cell membrane for secretion. A. shown is the live cell staining of unfixed tunicamycin (Tuni)-treated (24 h) and untreated HEK293 cells expressing Myc/His-tagged QSulf1 (QS-myc) with Cy3-conjugated anti-Myc antibody. FITC-conjugated lectin staining showed the integrity of the cell membrane and cell-surface HS. B. shown is the permeabilized cell staining of fixed tunicamycin-treated (24 h) and untreated cells expressing Myc/His-tagged QSulf1 and the control pcDNA3.1 plasmid. Calreticulin served as an ER marker. C. shown are the results from Western blot (WB) analysis of QSulf1 in the cell lysates and secreted into the medium of control or tunicamycin-treated HEK293T cells stably transfected with Myc/His tagged-QSulf1. Cells were treated with tunicamycin for 24 h before harvesting. The medium was concentrated 10-fold. Blots were probed with anti-Myc antibody to detect QSulf1 and with anti-actin antibody to monitor gel loading. D. QSulf1 detected in Fig. 5A was densitometrically quantified using Multi Analyst software, and the results are presented in the form of a bar graph, *, p < 0.05 relative to the untreated control. QSulf1 Secreted, QSulf1 protein secreted into the medium; QSulf1 Cellular, intracellular QSulf1 protein. All QSulf1 protein expression was a total of both glycosylated and unglycosylated QSulf1, indicated by blue and red arrowheads, respectively.

Glycosylation of QSulf1 Is Required for Its Membrane Targeting and Secretion in HEK293T Cells—N-Glycosylation often regulates intracellular transportation and secretion of glycoproteins. To test the role of glycosylation in the membrane targeting of Sulf, we investigated the intracellular localization of stably transfected QSulf1 in HEK293T cells treated with tunicamycin. Glycosylation initiates in the ER and is completed in the Golgi apparatus as proteins are trafficked to the membrane (20). We observed that deglycosylated QSulf1 was not trafficked to the cell surface, as revealed by the absence of QSulf1 on the cell surface of tunicamycin-treated cells using a live staining protocol (Fig. 6A). Tunicamycin treatment did not disrupt the membrane trafficking of all membrane proteins, as FITC-conjugated lectin live labeling of cell-surface HS was not affected in tunicamycin-treated cells (Fig. 6A).

It is significant that partial removal of N-linked glycosylation blocked QSulf1 enzymatic activity and heparin binding (Fig. 5, C and D). After 12 h of N-glycanase treatment under nondenaturing conditions, the majority of QSulf1 was largely deglycosylated, as evidenced by its reduced molecular mass of 109 kDa; this deglycosylated QSulf1 had no HS 6-O-endosulfatase activity and had reduced heparin binding compared with control QSulf1 without N-glycanase treatment, which was active and exhibited significant binding to heparin after 12 h of incubation (Fig. 5, C and D). The buffer for native N-glycanase digestion did not maintain QSulf1 enzymatic activity after 24 h (Fig. 5C). These findings therefore establish that glycosylation of QSulf1 is essential to maintain its enzymatic function, independent of biosynthesis.

Glycosylation of QSulf1 Is Required for Its Membrane Targeting and Secretion in HEK293T Cells—N-Glycosylation often regulates intracellular transportation and secretion of glycoproteins. To test the role of glycosylation in the membrane targeting of Sulf, we investigated the intracellular localization of stably transfected QSulf1 in HEK293T cells treated with tunicamycin. Glycosylation initiates in the ER and is completed in the Golgi apparatus as proteins are trafficked to the membrane (20). We observed that deglycosylated QSulf1 was not trafficked to the cell surface, as revealed by the absence of QSulf1 on the cell surface of tunicamycin-treated cells using a live staining protocol (Fig. 6A). Tunicamycin treatment did not disrupt the membrane trafficking of all membrane proteins, as FITC-conjugated lectin live labeling of cell-surface HS was not affected in tunicamycin-treated cells (Fig. 6A).

N-glycosylation of QSulf1 is required for its activity, we isolated QSulf1 from overexpressing HEK293T cells and digested QSulf1 with N-glycanase under nondenaturing conditions over a course of 1 h to 5 days. We observed a gradual transit of QSulf1 molecular mass from 125 to ~109 kDa as early as after 1 h of treatment, and a majority of QSulf1 migrated at ~109 kDa on SDS gels after 12–16 h (Fig. 5, A and B). N-Glycanase digestion under native conditions after 5 days reduced the molecular mass of QSulf1 to ~104 kDa; however, extended digestion did not reduce the molecular mass of QSulf1 to the 100-kDa unglycosylated form (Fig. 5A), indicating that some N-linked glycosylation sites are inaccessible to N-glycanase under native conditions likely because QSulf1 is folded.

Notably, immunostaining under permeabilized conditions revealed that unglycosylated QSulf1 accumulated in the ER and colocalized with the ER-specific marker calreticulin (Fig. 6B). Furthermore, tunicamycin also significantly blocked the low level secretion of QSulf1 into the culture medium (Fig. 6, C and D). Therefore, N-glycosylation of QSulf1 is essential for its cell-surface targeting, extracellular matrix localization, and secretion.

Proteins that are not properly glycosylated may be subject to degradation in the cytosol. To investigate whether N-glycosylation of QSulf1 also affects the cytosolic and membrane distribution of QSulf1 protein, we fractionated cell lysates of QSulf1-expressing HEK293T cells into cytosolic, membrane, and cytoskeletal fractions using the FractionPREP fractionation kit. We found that QSulf1 was associated predominantly with the membrane, with low levels of cytosolic and cytoskeletal localization (Fig. 7). Tunicamycin did not alter the general subcellular association of QSulf1. In addition, tunicamycin did not increase the fraction of cytosolic QSulf1, suggesting that unglycosylated QSulf1 is not subject to major degradation.
Glycosylation Is Required for Membrane Targeting of Endogenous mSulf1—To test whether endogenous Sulfs require N-glycosylation for membrane targeting, we isolated primary rhabdomyosarcoma cells from rhabdomyosarcoma formed in Patched+/−/− mice (17). These primary rhabdomyosarcoma cells expressed mSulf1 mRNA as determined by reverse transcription-PCR (Fig. 8A) and immunostaining using anti-MSulf1, anti-c-Met, and anti-syndecan-4 antibodies (Fig. 8B). Live staining of these primary cells detected the expression of c-Met and syndecan-4 on the cell surface. The extracellular localization of MSulf1 was demonstrated by its colocalization with syndecan-4 and HS labeled by FITC-conjugated wheat germ lectin under nonpermeabilizing staining conditions (Fig. 8, B and C). Upon tunicamycin treatment, no MSulf1 was detected on the cell surface, although HS staining was unaffected (Fig. 8C). These results demonstrate that N-glycosylation is required for targeting endogenous Sulf on the cell surface.

DISCUSSION

Here, we have demonstrated that QSulf1 is an N-linked glycosylated protein. Removal of N-glycosylation of QSulf1, by either tunicamycin or N-glycanase, disrupts QSulf1 heparin binding and 6-O-endosulfatase activity. Tunicamycin treatment also abolishes cell membrane targeting of QSulf1, leading to intracellular accumulation of unglycosylated QSulf1. Together, our results have established N-glycosylation as an essential post-translational modification mechanism for the function of Sulfs as extracellular signaling regulators. Human Sulfs also undergo N-glycosylation, providing N-glycosylation as a potential drug target for blocking Sulf-dependent tumor growth.

Domain mapping studies have localized five functional N-glycosylation sites within the N-terminal enzymatic domain and two in the C-terminal domain. Although the HD has two predicted N-glycosylation sites, these two sites were not functional in our experiments. QSulf1 appears to have simple and not branched N-linked glycochains, as inhibitors that block branched glycosylation did not cause a shift in the molecular mass of QSulf1 (supplemental Fig. 1D). The specific functions of N-glycosylation in the control of Sulf enzymatic properties...
and cell-surface localization remain unknown. One possibility is that N-glycosylation of Sulfs is required to maintain proper protein folding and conformation to generate an active substrate-binding and catalytically active structure to enable Sulf endosulfatase activity. An alternative but not mutually exclusive possibility is that the glycochains function directly to control enzymatic activity, e.g. through binding to and presenting the heparin substrates to the catalytic site. Further studies are required to more fully understand the structural requirement of N-linked glycosylation for Sulf function.

Our tunicamycin inhibition studies provide new evidence that glycosylated QSulf1 dynamically turns over on the cell membrane. After 24 h of tunicamycin treatment, the majority of glycosylated QSulf1 is lost from cell surface, either through degradation or secretion. Such a turnover could provide a mechanism for the temporal control of Sulf in cell-surface and matrix signaling in embryonic and adult progenitor lineages (2). It remains to be determined which N-glycosylated sugar chains have any functional role in QSulf1 once it is localized on the cell surface, such as to control the interaction of QSulf1 with its HS substrate or with HS proteoglycans. Future studies of the role of specific N-glycosylation sites in the N- and C-terminal domains may provide further insights into the regulatory functions of glycosylation in QSulf1 trafficking and signaling functions.

Acknowledgments—We thank Drs. Pravir Kumar and Jennifer Chen for discussion and expert advice.

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