Substrate Specificity and Domain Functions of Extracellular Heparan Sulfate 6-O-Endosulfatases, QSulf1 and QSulf2

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The extracellular sulfatases (Sulfs) are an evolutionarily conserved family of heparan sulfate (HS)-specific 6-O-endosulfatases. These enzymes remodel the 6-O-sulfation of cell surface HS chains to promote Wnt signaling and inhibit growth factor signaling for embryonic tissue patterning and control of tumor growth. In this study we demonstrate that the avian HS endosulfatases, QSulf1 and QSulf2, exhibit the same substrate specificity toward a subset of trisulfated disaccharides internal to HS chains. Further, we show that both QSulfs associate exclusively with cell membrane and are enzymatically active on the cell surface to desulfate both cell surface and cell matrix HS. Mutagenesis studies reveal that conserved amino acid regions in the hydrophilic domains of QSulf1 and QSulf2 have multiple functions, to anchor Sulf to the cell surface, bind to HS substrates, and to mediate HS 6-O-endosulfatase enzymatic activity. Results of our current studies establish the hydrophilic domain (HD) of Sulf enzymes as an essential multifunctional domain for their unique endosulfatase activities and also demonstrate the extracellular activity of Sulfs for desulfation of cell surface and cell matrix HS in the control of extracellular signaling for embryonic development and tumor progression.

Heparan sulfate proteoglycans (HSPGs) have essential roles in extracellular signaling pathways through their binding to signaling ligands and functions as co-receptors (1–3). HSPGs contain a protein core and covalently linked linear heparan sulfate (HS) chains of repeating uronic acid-glucosamine disaccharide units. The uronic acid unit may be either glucuronic acid or its C5 epimer iduronic acid. HS chains are heterogeneous with structurally diverse and functionally distinct sequences along the chains based on variable patterns of N-acetyl, N-sulfate, and O-sulfate groups (4–6). Sulfation occurs in clusters creating highly sulfated N-sulfate domains flanked by alternating N-sulfated and N-acetylated disaccharide units (N-acetyl/N-sulfate domains) that separate N-sulfate domains from the unsulfated N-acetyl domains. The 6-O-sulfation pattern along HS chains is tissue-specific and dynamically regulated to modulate HSPG-dependent extracellular signaling (7–9).

We have identified a novel family of extracellular HS 6-O-sulfatases (Sulfs) that modify the 6-O-sulfation states of cell surface HSPGs to function as extracellular signaling regulators (10). QSulf1, first identified in a screen for Hedgehog target genes, is dynamically expressed in muscle and neural progenitors in embryos as well as in adults (10). Orthologues of QSulf1 have been identified in Caenorhabditis elegans, Drosophila, zebrafish, chick, mouse, and human, and a closely related QSulf2 has been identified in vertebrates (11–13). QSulf1 has four distinct and evolutionarily conserved structural domains: an N-terminal signal peptide that directs QSulf1 secretion onto the cell surface, a catalytic domain, a central hydrophilic domain required to associate QSulf1 on the cell surface, and a C-terminal domain (10).

Sulf function as cell surface regulators of HSPG-dependent extracellular signaling (10) and can act as tumor suppressors (14, 15). Antisense inhibition of QSulf1 expression in the embryo blocks Wnt-dependent gene expression in the embryonic somites, and QSulf1 overexpression in C2C12 myoblast cells enhances Wnt signaling (10). QSulf1 activity decreases the binding affinity between HS and the Wnt ligand to promote the binding of Wnt ligand to Frizzled receptor for signaling (16). QSulf1 also functions as a negative regulator of FGF signaling and FGF2-dependent angiogenesis by disrupting the FGF2-FGFR1-heparin ternary complex formation (17). QSulf1 over-expression in human primary tumor cell lines also inhibits the signaling activities of HGF and HS-dependent epidermal growth factor to repress tumor growth and cell migration (14, 15). Furthermore, QSulf1 modulates the binding affinity between BMP inhibitor Noggin and HS (18). Importantly, QSulf1 expression in muscle and neuronal lineages is dependent on Sonic hedgehog signaling during embryogenesis (10). In specific tumors, QSulf1 expression is absent, even though Sulf1 is expressed in normal progenitor lineages thought to give rise to these tumors (14, 15).

The signaling functions of Sulfs are based on their unique activities as HS 6-O-endosulfatases to remodel that enzymatically remove 6-O-sulfate groups from a subset of internal trisulfated disaccharides located in internal sulfated domains of HS chains on the cell surface (11, 16, 18). By contrast, the lysosomal GlcNR6Sase has exosulfatase activity that removes the 6-O-sulfate groups from terminal glucosamine residues of oligosaccharide intermediates during lysosomal HS catabolism (19). Sulf1 and GlcNR6Sase share extensive sequence homology within their enzymatic and C-terminal domains (10). However, QSulf1 has a centrally located hydrophilic domain (HD) that is required for associating QSulf1 on the cell surface.

In this study we have investigated the cell surface association, endosulfatase activity, and substrate specificity of QSulf1 and QSulf2 and the essential roles of the HD domain in QSulf enzymatic properties. We report that QSulf1 and QSulf2 both function as HS 6-O-endosulfatase with identical HS substrate specificities. Our findings also provide evidence that QSulf1 and QSulf2 are enzymatically active and function on the cell surface to modify the 6-O-sulfation states of cell surface and matrix HS, supporting the conclusion that Sulf enzymes function dynamically on the cell surface. Finally, we show that the conserved sequences in QSulf1...
and Sulf2 HD domains have multiple functions in GAG substrate binding, cell surface association, and endosulfatase activity.

**EXPERIMENTAL PROCEDURES**

Cloning of QSulf2 cDNA—A stage 12 quail cDNA library was screened under low stringency conditions using a cDNA probe of the QSulf1 enzymatic domain. Five positive clones were identified, including one 4.3-kb cDNA clone that was fully sequenced and contains the full-length QSulf2 cDNA (GenBank™AY766468). The QSulf2 cDNA was cloned by PCR into a pAG-His-Myc (C-terminal tag) mammalian expression vector. The hydrophilic domain of QSulf2 (QSulf2HD) contains conserved sequences (QSulf2HDC) and a not so conserved middle subdomain. The boundary of the middle subdomain was determined by a comparison with the Sulf2 gene in human and mouse in which the middle subdomain was encoded by two adjacent exons. An overlap extension PCR protocol was used to generate various deletions in QSulf2HD, including deletions of the middle domain, the conserved C-terminal sequences, and a cluster of 14 basic amino acid residues (C14). QSulf2HD, HDC, HDS, and HDAC14 were cloned into pAG3-Myc with an N-terminal secretion signal peptide and a Myc tag. Primers used in the studies are as follows: for cloning of QSulf2 into pAG-His-Myc expression vector, forward primer 5'-GGTACATCCGACGCAATGTCCAGAGGGCCG-3' and reverse primer 5'-CTGGTCTTCTGTGCTGTG-3' and reverse primer 5'-ACACAAAAACGAAATGCATAAAGAGGGG-3'; for deletion of QSulf2HD, forward primer 5'-CTAGTGGAGAGGC-TGTTCTTCTGTGCTGTG-3' and reverse primer 5'-ACACAAAAACGAAATGCATAAAGAGGGG-3'; for deletion of the conversed fragment in QSulf2HD, forward primer 5'-GGTACATCCGACGCAATGTCCAGAGGGCCG-3' and reverse primer 5'-CTGGTCTTCTGTGCTGTG-3' and reverse primer 5'-ACACAAAAACGAAATGCATAAAGAGGGG-3'; for deletion of the middle domain of QSulf2HD, forward primer 5'-GCTTTGAGAGGTCGTTTGCAAGGTGTTTACCCTGCATGAAATGTTTA-3' and reverse primer 5'-CTGCAGGATGTAACACCTGCCAAGCTGCTCACAAAGCTGCT-3'; for cloning of QSulf2 HD into pAG3-Myc expression vector, forward primer 5'-GGTACATCCGACGCAATGTCCAGAGGGG-3' and reverse primer 5'-CTGGTCTTCTGTGCTGTG-3'; and reverse primer 5'-ACACAAAAACGAAATGCATAAAGAGGGG-3'; for deletion of basic amino acid residues at the C terminus of QSulf2HD, forward primer 5'-TGTTCTGCTTCAAGAAAGAAAACACGACCGTGCACG-3' and reverse primer 5'-GCACGGTTCGTTGTTTGCCTCTCGAAGCACACACACACACG-3'.

The PCR products were cloned into T-easy vector (Promega), sequenced, and subsequently inserted into pAG-Myc expression vector. The amino acid sequences of QSulf2, Msulf2, Hsulf2, and QSulf1 were compared and aligned using MacVector.

**Cell Culture and Transfection**—293T cells were maintained in a 100-mm dish in culture medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum (Hyclone) + 1% antibiotics) and transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 48 h, cells were lysed in 500 μl of 0.25% Triton X-100 with protease inhibitor cocktails (Roche Applied Science). 293T cell lines that stably express QSulf1 or QSulf2 were selected by 200 μg/ml hygromycin B (Sigma). To analyze protein secretion, cells were transfected with Sulf2 HD. The conditioned medium was then collected and concentrated 50-fold using a Centricon-10 spin column before Western blotting.

**Immunocytochemistry**—Cells were plated on precleared coverslips, transfected, and then probed for immunocytochemistry. A live staining protocol was used to detect proteins on the cell surface, by which the transfectant cells were incubated with primary antibody in the culture medium for 2 h at 4 °C. Cells were washed twice with PBS before fixation with 4% paraformaldehyde/PBS. After washing, cells were incubated with appropriate secondary antibody in antibody dilution buffer (PBS, 0.1% Triton X-100, 20% goat serum) for 1 h at room temperature. Cells were then washed with PBS and mounted for fluorescent microscopy. Antibodies include anti-His tag (1:300, Clontech), anti-Myc (9E10, 1:400), goat anti-rabbit Cy2 (1:500, Molecular Probes), and goat anti-mouse Cy3 (1:500, Molecular Probes).

**Subcellular Fractionation**—Stable QSulf-expressing 293T cell lines were grown on a 100-mm culture dish until confluency. After rinsing with PBS, cells were scraped from the plate and collected by centrifugation. Cell pellets were resuspended in 500 μl of hypotonic Tris buffer (20 mM Tris-HCl, pH 7.4) and lysed by passing through a 27-gauge needle 40 times with the presence of protease inhibitors (Roche Applied Science). The cell lysate was centrifuged for 5 min at 14,000 × g to remove the nucleus. The supernatant was separated into a soluble cytosol portion and an insoluble membrane-bound portion by centrifuging for 30 min at 100,000 × g. The insoluble membrane-bound portion was resuspended in a Triton cell lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 15% glycerol) and then further separated into a Triton-soluble portion and a Triton-insoluble portion by a second 30-min centrifugation at 100,000 × g. The Triton-insoluble portion was dissolved in SDS lysis buffer containing 0.5% SDS, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA.

**Western Blot Analysis**—Proteins in cell extracts (20 μg), concentrated condition medium, or on anti-Myc-agarose beads were separated on 10% SDS-polyacrylamide gel and transferred to a Hybond-P membrane. The membrane was soaked in 5% nonfat dry milk in PBST (PBS + 0.1% Tween 20) for 1 h at room temperature to block unspecific binding of the antibody. The membrane was probed with the anti-His antibody (1:2000) for 2 h at room temperature, washed with PBST, and incubated with horse radish peroxidase-conjugated anti-rabbit secondary antibody (1:5000, Jackson Immunolaboratories) for 1 h at room temperature. To detect the anti-Myc IgG on agarose beads, the blot was incubated directly with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000; Jackson Immunolaboratories). The membrane was subsequently washed, and signals were detected by ECL (Amersham Biosciences).

**Purification of Heparan Sulfate from 293T Cells**—[35S]GAGs were prepared by metabolically labeling 293T cells for 5 h in F12 medium containing 1% FBS and 100 μCi/ml 35SO4. Cells were subsequently lysed in hypotonic 0.25% Triton X-100 in H2O, and the cell lysate was digested with protease K (10 μg/ml) at 55 °C overnight to degrade proteins. After boiling for 10 min to inactivate protease K, cellular GAGs were precipitated with 3 volumes of 100% ethanol and 10 μg of dermatan sulfate at −20 °C. The precipitate was collected by centrifugation, washed with 70% ethanol, air-dried, and dissolved in H2O. GAGs were digested with 0.2 unit of chondroitinase ABC (Seikagaku) to degrade chondroitin sulfate followed by HS purification with a 5-KDa filter unit to remove low molecular mass degraded products.

**Heparan Sulfate Binding Assays**—The QSulf2HD, QSulf2HDC, and QSulf2HDCΔC14 was transiently expressed in 293T cells and subsequently purified from cell lysates (500 μl) with 25 μl of anti-Myc beads. The truncated HD fragments migrated at the same position as anti-Myc antibody on a SDS-polyacrylamide gel, which made it difficult to assay the amounts of HD fragments on anti-Myc beads. Therefore, the cell lysate was in large excess to the binding sites on anti-Myc beads to guarantee the maximum binding of the expressed GAGs.
protein. After washing three times with PBS, the anti-Myc beads with bound QSulf2HD fragments were aliquotted into 10 μl and incubated with 5 μl of [35S]HS ( ~2 × 10^7 cpm) at room temperature for 1 h. The anti-Myc beads were subsequently washed with ice-cold PBS, and the 35S radioactivity retained on the beads was quantified with Beckman scintillation counter.

**Sulfatase Enzymatic Assay**—Stable QSulf-transfected 293T cells were lysed in hypotonic 0.25% Triton X-100 in H2O with protease inhibitor mixture. QSulf enzymes were purified with anti-Myc beads from the cell lysate. To strip Sulf protein from the cell surface, cells were rinsed with ice-cold PBS followed by 1M NaCl for 5 s. QSulf enzymes present in high salt washes were concentrated 20-fold by centricon-1 and dialyzed in TBS buffer before the enzymatic assay. For the endosulfatase activity assay, 35SO4-labeled GAG substrate (10 μl) was digested by Sulf enzyme (50 μl) at 37 °C overnight in a 200-μl volume containing 50 mM Tris-HCl buffer (pH 7.5) and 50 mM MgCl2. The released SO4 was collected by centrifuging the reaction at 14,000 rpm for 15 min using the 5-kDa filter unit, and the 35S radioactivity in the flow-through was subsequently quantified with Beckman scintillation counter.

**Structural Analysis of 35S-Labeled Cellular GAGs**—HS disaccharides were generated by deaminative cleavage of 35S-labeled HS and subsequently resolved by HPLC anion exchange chromatography (20). To determine the disaccharide composition of chondroitin sulfate, purified 35S-labeled chondroitin sulfate chains were degraded by digestion with 0.1 unit of chondroitinase ABC (Seikagaku) in 40 μl of 0.05 M Tris-HCl, pH 8.0, containing 0.03 M sodium acetate and 0.1 mg of bovine serum albumin. After incubation for 15 h at 37 °C, the digest was passed through a Superdex 30 (Amersham Biosciences) column, equilibrated with 0.5 M NH4HCO3. Disaccharides were recovered, freeze-dried, and fractionated further by descending paper chromatography conducted on Whatman No. 3MM paper in acetic acid/n-butanol/1 M ammonia (3:2:1, v/v). After 32 h, the paper strips were dried, cut into 1-cm segments, and analyzed for radioactivity by liquid scintillation counting. The radioactivity in each disaccharide product was calculated as the percentage of total radioactivity of HS or chondroitin sulfate.

**Preparation and Digestion of Extracellular Matrix (ECM) Coated on the Culture Plate**—293T cells were metabolically labeled with 35SO4 for 5 h in a 24-well plate. Cells were lysed for 3 min at room temperature by
Extracellular Localization and Enzymatic Activity of QSulfs

incubating the cells with a PBS solution containing 0.5% Triton X-100, 20 mM NH₄OH (21, 22). The ECM-coated wells were rinsed with PBS three times before the QSulf-expressing cells were plated. Cells were cultured overnight in the ECM-coated wells, and the medium was quantified for released [35S] radioactivity from ECM.

RESULTS

Characterization of QSulf2, a Second Member of the Avian Extracellular Endosulfatase Family—We cloned QSulf2 as a 4.36-kb cDNA by screening an embryonic quail cDNA library using the enzymatic domain of QSulf1 as a probe. QSulf2 is an 879 amino acid protein with a predicted molecular mass of 98 kDa. QSulf2, like other identified extracellular sulfatases, has four structural domains including an N-terminal signal peptide, an enzymatic domain, a hydrophilic domain, and a C-terminal domain (Fig. 1, A and B). The sequence comparison of QSulf2 to other extracellular sulfatases reveals extensive homology along the entire length of the protein (Fig. 1, A and C). The enzymatic domain of QSulf2 (red underline) is highly conserved across species and is 95% homologous to the enzymatic domain of QSulf1. Importantly, the enzymatic domain of QSulf2 includes two signature sequences, including an essential Cys residue in the catalytic site that is post-translationally converted to formylglycine for sulfatase enzymatic activity (Fig. 1A, red star). The hydrophilic domain (blue underline) of QSulf2 (QSulf2HD) shares an overall 86% homology with the HD domains of mammalian Sulf2 orthologues (Fig. 1A) and also between Sulf1 and Sulf2 (Fig. 4A). The amino acid sequences at the N and C termini of the HD are largely conserved, suggesting the functional significance of these sequences. By contrast, the internal sequences within the hydrophilic domain (Fig. 1A, double blue underline) encoded by exons #10 and #11 in mouse and human Sulf genes (double blue underline), include the majority of the sequence divergence in vertebrate Sulf2 proteins (Fig. 1A) and also between Sulf1 and Sulf2 (Fig. 4A).

QSulf2 and QSulf1 Have Identical HS Substrate Specificities—To investigate QSulf2 substrate specificities, QSulf2 cloned into the pAG expression vector with a C-terminal Myc and His tag was expressed in a stably transfected 293T cell line. QSulf2 protein was affinity-purified using anti-Myc antibody conjugated to the agarose beads. Cellular [35S]GAG substrate was prepared by metabolically labeling 293T cells. After enzymatic digestion, QSulf2 and QSulf1 each released ~5% 35S radioactivity from [35S]HS substrate, whereas control extracts prepared from cells transfected with empty vector or enzymatically inactive QSulf1(C-A) released background levels of 35S radioactivity (Fig. 2A), consistent with their activities as endosulfatases (16). QSulf2, like QSulf1, also was inactive on N-acetylgalactosamine 6-O-sulfate monosaccharide substrate (data not shown), further supporting the conclusion that QSulf2 is an HS endosulfatase.

To investigate QSulf2 substrate specificity, QSulf2-digested cellular [35S]HS were subjected to disaccharide compositional analysis by deaminative cleavage followed by HPLC (20). Four major [35S]disaccharide products were resolved, including GlcA-GlcNS6S, IdOa-GlcNS6S, IdOa2S-GlcNS, and IdOa2S-GlcNS6S (Table 1). Each disaccharide was quantified by measuring the retained 35S radioactivity, expressed as the percentage of total 35S radioactivity of all four disaccharides. QSulf2 primarily removes 6-O-sulfates from trisulfated IdoA2S-GlcNS6S, as reflected in a decrease of IdOa2S-GlcNS from 36.4 to 26.6% and a parallel increase of IdOa2S-GlcNS6S, from 48.8 to 59.6%. QSulf2 was active on chondroitin sulfate-derived substrates (Table 1). Therefore, QSulf2, like QSulf1, is an HS-specific 6-O-endosulfatase with substrate specificity for a subset of trisulfated disaccharides residues within the HS chains.

QSulf1 and QSulf2 remove a similar fraction of 6-O-sulfates from HS, suggesting that both QSulfs have restricted substrate specificities for 6-O-sulfates. To distinguish whether QSulf2 and QSulf1 recognize the same or different subsets of 6-O-sulfates from trisulfated disaccharide residues on cellular HS chains, cellular [35S]GAGs were sequentially digested with QSulf1 and QSulf2, and vice versa. Sulfate release was monitored after each digestion. We found that [35S]GAGs digested by QSulf1 were not further desulfated by a subsequent QSulf2 treatment. Likewise, QSulf1 released no additional 35S radioactivity on or from [35S]GAGs predigested with QSulf2 (Fig. 3B), by contrast, QSulf1 or QSulf2 was fully active on cellular [35S]GAGs substrates that were predigested by enzymatically inactive QSulf1(C-A). Therefore, the enzymatic activities of QSulf1 and QSulf2 are restricted to the identical subset of trisulfated disaccharides along cellular HS chains, providing direct evidence that QSulf1 and QSulf2 have the same substrate specificity and redundant HS remodeling functions.

QSulf1 and QSulf2 Are Enzymatically Active on the Cell Surface—QSulf2, like QSulf1, is anchored on the cell surface, as detected by live staining of Myc-tagged protein, and in the intracellular endoplasmic reticulum and Golgi, as detected by immunostaining of permeabilized cells (data not shown). Also like QSulf1, QSulf2 protein is not secreted and released as a soluble enzyme into the tissue culture medium of transfected cell cultures (data not shown). To further investigate the association of QSulf1 and QSulf2 with cell membranes, 293T cells stably
expressing QSulf1 or QSulf2 were lysed in a hypotonic buffer followed by ultracentrifugation to separate soluble versus membrane fractions of the cell lysates. Both QSulf1 and QSulf2 were detected in the cell membrane fraction but not in the soluble fraction (Fig. 3A). Significantly, a large fraction of QSulf is localized to the Triton-insoluble membrane fraction, which is enriched in cytoskeletal components and lipid rafts (23), suggesting that QSulf is localized in the lipid raft domain of the cell membrane.

To test whether QSulfs on the cell surface are functional in HS 6-O-desulfation, we isolated membrane-bound QSulfs using a transient high

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**Table 1: Effects of QSulf2 on 35S-labeled cellular GAGs**

| Deamination products (% O-[35S]disaccharide) | Heparan sulfate | Chondroitin sulfate | Heparan sulfate | Chondroitin sulfate |
|---------------------------------------------|----------------|--------------------|----------------|--------------------|
| GlcA-aManb6GlcNS GMS                        | 9.2 ± 1.1       | 10.0 ± 0.7         | 1.1 ± 1.1       | 18.0 ± 0.1         |
| IdoA2-aManb6GlcNS IMG                       | 4.6 ± 2.0       | 4.1 ± 0.3          | 2.0 ± 0.1       | 18.5 ± 0.5         |
| IdoA2-aManb6GlcNS ISM                       | 59.6 ± 4.2*     | 48.8 ± 2.9*        | 4.2 ± 0.1       | 63.5 ± 0.7         |
| IdoA2-aManb6GlcNS ISMS                      | 26.6 ± 2.4*     | 36.4 ± 3.4*        | 2.4 ± 0.1       | 36.4 ± 3.4*        |

**Digestion products (% -[35S]disaccharide)**

| Digestion products (% -[35S]disaccharide) | Heparan sulfate | Chondroitin sulfate |
|-------------------------------------------|----------------|--------------------|
| ΔDidS                                     | 18.5 ± 0.7     | 18.0 ± 0.1         |
| ΔDiiS                                     | 19.0 ± 0.1     | 18.5 ± 0.5         |
| ΔDiiS                                     | 62.5 ± 0.7     | 63.5 ± 0.7         |

*a p < 0.01 (Student’s t test).*
FIGURE 4. The hydrophilic domain of QSulf2 is required for cell surface association, HS binding, and the endosulfatase activity. A, the amino acid sequence alignment of QSulf1 HD and QSulf2 HD. The variable middle domain is shown with blue underline. Heparin-binding motifs were indicated by a solid upper line for QSulf1HD and dashed underline for QSulf2HD. The cluster of 14 basic amino acid residues is marked by a red underline.

B, scheme of various deletions in QSulf2HD. C, Western blot analysis of the expression of HD, HDC, HDS, and HD/C14. Most of the expressed polypeptides are present in the cell lysate, whereas the conditioned (con.) medium contained mostly degraded polypeptides. Ctrl, control.

D, cell surface staining of QSulf2HD, HDC, HDS, and HD/C14. 3T3 cells were transfected with pAG-N-Myc expression vector containing various HD sequences before cells were live-stained with anti-Myc antibody. QSulf2HD, HDC, and HD/C14 associate extensively with the extracellular matrix, similar to the surface staining of QSulf2. In contrast, HDS showed...
Salt wash that strips off the cell matrix proteins without lysing the cells (22). 293T cells remained viable after this wash procedure. The extracted proteins were concentrated and dialyzed in TBS before Western blot analysis and sulfate release assays with cellular [35S]GAG substrate. QSulf protein recovered from extracellular membranes was enzymatically active (Fig. 3B), establishing that cell membrane-associated QSulfs are functional enzymes.

We have shown previously that a Golgi-trapped QSulf1 remodels HS 6-O-sulfation on the cell surface, suggesting that QSulfs can function intracellularly during HS biosynthesis in Golgi. However, the question remained whether the cell-autonomous remodeling of HS sulfation is because of QSulf1 activity on the cell surface or in the Golgi. Additionally, whether cell surface QSulf can act in trans to desulfate HS in the matrix was not known. To investigate whether QSulfs are active on the surface of living cells, we prepared ECM-coated plates coated with [35S]radiolabeled GAG substrates by metabolically labeling the 293T cells with [35SO₄] and subsequently lysing the labeled cells, leaving ECM bound to the surface of the plate (21, 22). 293T cell lines that stably express either QSulf1, QSulf2, or enzymatically inactive QSulf1(C-A) were then plated on the [35S]radiolabeled ECM, cultured overnight, and assayed for [35S]release into the culture medium. Cells expressing QSulf1 or QSulf2 actively released [35S]radioactivity (Fig. 3C), whereas control cells expressing enzymatically inactive QSulf1(C-A) released only background levels of [35S]radioactivity. Furthermore, cells expressing endoplasmic reticulum-targeted QSulf1-ER or Golgi-targeted QSulf1-Golgi also did not release [35S]radioactivity from the ECM (Fig. 3D), even though these intracellularly targeted QSulf1-ER and QSulf1-Golgi are enzymatically active (16). These findings, therefore, provide direct evidence that QSulfs on the cell surface are functional to dynamically modify the 6-O-sulfation patterns of both cell surface and extracellular matrix-localized HS.

The Hydrophilic Domain of QSulf2 Is Required for the Endosulfatase Activity—The extracellular sulfatase family members have a characteristic HD that is absent from the lysosomal GlcNR6Sase. Deletion studies show that the HD is required for anchoring QSulf1 and QSulf2 on the cell surface (Ref. 10 and data not shown). Directed deletion studies were used to localize specific sequences in the HD required for cell surface association and to test the function of HD domain in substrate binding and enzymatic activity. The HD is encoded by six exons in human and mouse Sulf genes and has conserved N- and C-terminal amino acid sequences in Suls in different vertebrate species (Fig. 1A) and in QSulf2HD and QSulf1HD (Fig. 4A). These conserved HD sequences (HDC) contain abundant charged amino acid residues including a cluster of basic arginine and lysine residues near the C terminus (Fig. 4A, red underline). The middle subdomain of QSulf2HD, encoded by two adjacent exons in human and mouse, is more divergent (Fig. 1A, double blue underline) and includes Sulf2-specific sequences conserved in different vertebrate Sulf2 proteins but is much less conserved between Sulf1 and Sulf2 (compare homologies in Fig. 1A with Fig. 4A).

To further investigate HD functions, we generated expression constructs of different HD domains by PCR, including the entire QSulf2 HD, the conserved HDC, the divergent HDS containing mostly the middle domain, and HDCΔ14 with a 14-amino-acid deletion of a highly basic sequence in the HD conserved region (Fig. 4B), which were cloned into pAG expression vector with a N-terminal signal peptide and a Myc tag. When expressed in 3T3 cells, the HD (47 kDa), HDC (30 kDa), HDS (28 kDa), and HDCΔ14 (45 kDa) polypeptides are largely present in the cell lysate but not in the conditioned medium of transfected cells (Fig. 4C). Several truncated forms of the various HD polypeptide are detected in the conditioned medium, which may be because of the cleavage by the furin-type proprotein convertase (11). The cell surface localization of various HD polypeptides was assayed by live staining (Fig. 4C) with the anti-Myc antibody. The HD, HDC, and HDCΔ14 associate extensively on the cell surface, whereas the HDS was poorly retained on the cell surface (Fig. 4D), establishing that the conserved hydrophilic domain sequence lacking the 14-amino-acid conserved cluster is sufficient for anchoring QSulf2 on the cell surface.

The HD of vertebrate Suls is highly basic (22% Lys and Arg residues), suggesting that HD may bind to sulfated GAGs, as a substrate binding domain. To test this possibility, HD, HDC, HDS, and HDCΔ14 were purified from transfected 293T cells by anti-Myc antibody conjugated to agarose beads. One of the IgG bands migrated at the same position as QSulf2HD on the SDS-polyacrylamide gel, making it difficult to directly assess the amount of various purified HD polypeptides. Instead, we used the amount of IgG light chain (~27 kDa) to control the amount of purified polypeptides on anti-Myc beads. Various HD polypeptides purified on the anti-Myc beads were subsequently incubated with purified cellular [35S]HS for 1 h at room temperature to assay binding. After washing with PBS, the [35S]radioactivity retained on anti-Myc beads was quantified (Fig. 4E). A significant amount of [35S]radioactivity was detected with HD, HDC, and HDCΔ14 on the anti-Myc beads but not with HDS and empty vector-transfected control. Similar binding results were observed with purified cellular chondroitin sulfate (data not shown). The binding of HS with HD sequences was competed by decasaccharide, heparin, and dermatan sulfate (data not shown). Therefore, the conserved sequences within the HD likely bind to negatively charged GAGs, which may serve not only as a mechanism to anchor the QSulf protein on the cell surface but also to bind GAG substrates for enzymatic digestion.

To investigate whether sequences in the HD are required for Sulf enzymatic activity, we generated various QSulf2 proteins with deletions of HD sequences and assayed the enzymatic activities of these truncated proteins by sulfate release with [35S]GAG substrates (Fig. 4F). QSulf2 with deletions of the HD (QSulf2ΔHD) or C-terminal conserved HD sequences (QSulf2ΔHDC and QSulf2ΔHDCΔ14) lacked 6-O-endosulfatase activity. By contrast, removal of the variable middle HDS (QSulf2ΔHDS) decreased activity by only one-third. Similar results were also obtained with comparable QSulf1HD deletions (data not shown). Together, these results indicate that HD domain is multifunctional, with conserved sequences required for cell surface association and GAG binding, as well as the enzyme activity of Suls.

**DISCUSSION**

In this study we have investigated and compared the substrate specificities, cell surface localization, and enzymatic activities of QSulf1 and QSulf2, avian Sulfs isomers. Our current study has established crucial roles of the hydrophilic domain for enzymatic activity, GAGs substrate binding, and cell surface localization of extracellular Sulfs. We also have shown that QSulf1 and QSulf2, a newly identified avian extracellular sulfatase, exhibit the same substrate specificity toward a subset of trisul-
fated disaccharides within the HS chains. Both QSulfs are enzymatically active on the cell surface, providing direct evidence that Sulfs remodel the HS 6-O-sulfation pattern both inside and outside of the cell for HS-dependent extracellular signaling.

Sequential digestion assays provide compelling evidence that QSulf1 and QSulf2 remove 6-O-sulfates from an identical subset of trisulfated disaccharides in HS chains, establishing that QSulf1 and QSulf2 have biochemically redundant functions. However, QSulf1 and QSulf2 are expressed in distinct embryonic tissues, and their expression is dynamically regulated in vivo. Furthermore, Sulf enzymes regulate a diversity of HS-dependent signaling pathways, indicating that Sulf1 and Sulf2 have distinct biological roles during embryogenesis and tumor growth. It also is notable that the HD domains of Sulf1 and Sulf2 have isomser-specific conserved sequence regions that could mediate different functions, modulating their interactions with cell surface proteoglycan and receptor complexes to target their activities to heparan substrates on the cell surfaces of expressing, or neighboring cells, or to the extracellular matrix.

Our findings also provide new evidence that Sulf enzymes are active in the extracellular environment on HS in extracellular matrix as well as HS on the cell surface, as well as on HS in the intracellular environment during HS biosynthesis in the Golgi (16). The major HS moiety in the extracellular matrix is present as perlecian, a secreted HSPG, suggesting that perlecians, in addition to glypicans (16), are substrates of Sulfs. Although Sulf enzymes can remodel the sulfation of HS in extracellular matrix, it remains to be determined whether Sulfs can modify the sulfation patterns of the HS of adjacent cells. The cell surface activity of QSulfs suggests its dynamic function as a cell surface regulator of receptor-ligand activity, but also provides a rationale for the design of specific drugs and blocking antibodies to inhibit or promote extracellular Sulf activity to control stem cell and tumor cell signaling and proliferation.

Our studies also demonstrate that the hydrophilic domain is a multifunctional domain of extracellular sulfatases. Importantly, the conserved sequences in the hydrophilic domain are crucial for its membrane association, HS substrate binding, and endosulfatase enzymatic activity. Nevertheless, the hydrophilic domain does not selectively bind to HS, indicating that the hydrophilic domain is not sufficient to control the heparan sulfate substrate specificity of Sulfs. The interaction between the hydrophilic domain and GAG likely contributes but is not essential to the membrane association of Sulfs. Our previous study has shown that QSulf1 remains anchored on the surface of CHO cells that are deficient in the biosynthesis of heparan sulfate and chondroitin sulfate (10), suggesting that Sulfs bind to other charged cell surface components such as dermatan sulfate to maintain its cell surface localization in GAG-deficient Chinese hamster ovary cells.

The HD domain has multiple functions for HS binding and enzyme activity. The conserved GAG-binding sequences in the hydrophilic domain are required for enzymatic activity. However, the GAG-binding capacity of HD alone is insufficient for enzymatic activity, as shown by the finding that the deletion of a cluster of conserved, highly basic 14 amino acid residues causes a loss of enzyme activity but not GAG binding. The specific functions of this 14-amino-acid domain are not yet known. However, one attractive model would be that the hydrophilic domain binds to HS substrates, presents the HS substrate to the catalytic domain and functions to define the unique 6-O-endosulfatase activity and specificity for selective trisulfated disaccharides. Future structural analysis of Sulfs will be required to fully understand the structure/function relationship of the hydrophilic and enzymatic domains to determine their endosulfatase activity and substrate specificity. The cell surface activity of Sulfs and the identification of the HD as a functional domain provide a basis for future studies to define Sulf HS substrates on cell surface and also to use Sulfs as drug targets for cancer therapy and stem cell engineering.

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