QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling

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The 6-O sulfation states of cell surface heparan sulfate proteoglycans (HSPGs) are dynamically regulated to control the growth and specification of embryonic progenitor lineages. However, mechanisms for regulation of HSPG sulfation have been unknown. Here, we report on the biochemical and Wnt signaling activities of QSulf1, a novel cell surface sulfatase. Biochemical studies establish that QSulf1 is a heparan sulfate (HS) 6-O endosulfatase with preference, in particular, toward trisulfated IdoA2S-GlcNS6S disaccharide units within HS chains. In cells, QSulf1 can function cell autonomously to remodel the sulfation of cell surface HS and promote Wnt signaling when localized either on the cell surface or in the Golgi apparatus. QSulf1 6-O desulfation reduces XWnt binding to heparin and HS chains of Glypican1, whereas heparin binds with high affinity to XWnt8 and inhibits Wnt signaling. CHO cells mutant for HS biosynthesis are defective in Wnt-dependent Frizzled receptor activation, establishing that HS is required for Frizzled receptor function. Together, these findings suggest a two-state “catch or present” model for QSulf1 regulation of Wnt signaling in which QSulf1 removes 6-O sulfates from HS chains to promote the formation of low affinity HS–Wnt complexes that can functionally interact with Frizzled receptors to initiate Wnt signal transduction.

Introduction

Heparan sulfate proteoglycans (HSPGs)* are major components of the extracellular matrix that regulate transmission of developmental signals and also are implicated in the pathophysiology of diseases, including cancer, in which signals and tissue interactions malfunction (Selva and Perrimon, 2001; Nybakken and Perrimon, 2002). HSPGs are classified as soluble and membrane-intercalated subtypes such as Glypicans and Syndicans, which are composed of a core protein decorated with covalently linked heparan sulfate (HS) chains (Bernfield et al., 1999). HS chains are polysaccharides that are synthesized in the Golgi apparatus and contain repeating disaccharide units of uronic acid linked to glucosamine (Bernfield et al., 1999; Prydz and Dalen, 2000). The disaccharide units are selectively sulfated at the N, 3-O, and 6-O positions of glucosamine and the 2-O position of uronic acid residues by actions of sulfotransferases in the Golgi apparatus. After biosynthesis, HSPGs are secreted to the cell surface or the extracellular matrix, where they have signaling and matrix functions (Bernfield et al., 1999; Nybakken and Perrimon, 2002). Cell surface HSPGs are also shedded and/or internalized by an endocytosis pathway involving HS degradation by catabolic enzymes, including exosulfatases for removal of terminal sulfates on sugar residues (Yanagishita and Hascall, 1992; Bai et al., 1997).

The extracellular signaling activities of HSPGs are mediated by their HS chains, which bind a diversity of developmental signaling ligands (Nybakken and Perrimon, 2002; Rapraeger, 2002). The sulfation states of HS chains influence their interactions with signaling molecules. FGF signal transduction is dependent on the sulfation of 2-O and 6-O positions on HS chains. The 2-O sulfation is required for bFGF binding to heparin and 6-O sulfation for bFGF-dependent dimerization and activation of the FGFR1 receptor, as revealed by both biochemical (Pye et al., 2000; Jemth et al., 2002) and the crystal structure studies of FGF–FGFR1–heparin ternary complexes (Schlessinger et al., 2000). Wnt (Wingless [Wg])...
signaling also is controlled by HS sulfation. The *Drosophila sulfatêles* gene encodes an HS N-deacetylase/N-sulfotransferase, and *sulfatêles* mutants are completely deficient in HS sulfation and have disrupted Wg signaling (Lin and Perrimon, 1999; Toyoda et al., 2000). Furthermore, chlorate, which is a metabolic inhibitor of HS sulfation, blocks Wnt (Wg) signaling in *Drosophila* and mammalian cultured cells (Reichsman et al., 1996; Dhoot et al., 2001). Therefore, the signaling activities of HSPGs in extracellular signaling are regulated by HSPG sulfation.

HS sulfation is dynamically regulated and tissue specific. In particular, the 6-O sulfates of HSPGs are precisely positioned, leading to microheterogeneity along the length of HS chains (Brickman et al., 1998; Merry et al., 1999; Safaiyan et al., 2000). Changes in HS PG 6-O sulfation have been correlated with regulatory changes in FGF signaling during neural development and tumor transformation (Brickman et al., 1998; Jayson et al., 1999). How the heterogeneous sulfation patterns of HSPGs are generated and dynamically maintained during the development has not previously been known. Most enzymes involved in Golgi-based HS biosynthesis and lysosomal HS degradation appear to be constitutively expressed in different tissues (Prydz and Dalen, 2000), and it has so far been difficult to pinpoint their roles as HS sulfation regulators. Furthermore, previously characterized HS sulfatases are exosulfatases that remove terminal sulfates from HS chains (Kresse et al., 1980; Raman et al., 2003) and, therefore, cannot generate intramolecular microheterogeneity of HS sulfation. Therefore, although HS sulfation is dynamically regulated to create HS microheterogeneity on HSPGs (Lindahl et al., 1998; Esko and Lindahl, 2001), mechanisms for regulation of HSPG sulfation remain unknown.

In this paper, we report on the biochemical and Wnt signaling activities of a novel extracellular sulfatase, QSLft, which is a candidate developmental regulator of HSPG sulfation in embryonic progenitor lineages (Dhoot et al., 2001). QSLft has an enzymatic domain homologous to lysosomal HS-specific GlcNR6Sase (GlcNR6Sase), which functions in the lysosomal degradation of HS. Unlike GlcNR6Sase, QSLft has an N2-terminal secretion signal peptide and hydrophilic domain for secretion and docking the cell surface. Homologues of QSLft have been identified in both vertebrates and invertebrates (Dhoot et al., 2001; Morimoto-Tomita et al., 2002; Ohno et al., 2002), and a second related family member, Sulf2, has been identified in mammals (Morimoto-Tomita et al., 2002) and birds (unpublished data). QSLft is essential for activation of the myogenic regulator MyoD for specification of muscle progenitors in embryonic somites and promotes Wnt-dependent signaling in myoblasts (Dhoot et al., 2001). Mutations that disrupt an essential N-formylglycine modification in the catalytic site block QSLft function in the Wnt signaling pathway, suggesting that QSLft functions as an enzymatically active sulfatase. We now show that QSLft is an HS-specific 6-O endosulfatase with a high degree of substrate specificity for 6-O-sulfated disaccharides of HS chains of HSPGs, including Glypican1, which is required for Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). QSLft localized on the cell surface or targeted in the Golgi apparatus is functionally active in remodeling the 6-O sulfation states of HSPGs on the cell surface and promotes Wnt signaling. Biochemical and cell biological studies of Wnt–HS binding and Frizzled receptor activity reveal that QSLft functions as part of a two-state “catch or present” mechanism to regulate Wnt signaling, specifically to modulate the binding affinity of Wnts to HS chains on HSPGs to promote the HS-mediated presentation of Wnt ligand to its Frizzled receptor to initiate signaling.

**Results**

**QSLft is an HS-specific sulfatase**

QSLft has sequence homology with the catalytic domain of GlcNR6Sase, a 6-O exosulfatase involved in the lysosomal catabolism of HS. Mutation of a critical Cys89 in the QSLft catalytic domain blocks its Wnt signaling activity, suggesting that QSLft is an enzymatically active sulfatase (Dhoot et al., 2001). To investigate the enzymatic activity of QSLft on sulfated glycosaminoglycan (GAG) substrates, 293 cells were metabolically labeled with [35S]SO4, and high molecular mass, 35S-labeled GAGs were isolated for enzymatic activity. Myc-tagged QSLft expressed by transient transfection of 293 cells was isolated and purified 15-fold by affinity purification on Myc beads, as determined by Western blot analysis (unpublished data). QSLft in cell lysates and Myc bead-purified preparations was incubated with [35S]GAGs and then assayed for [35S]SO4 release using a spin column method to monitor enzyme activity. As controls, cell extracts were prepared from 293 cells transfected with either empty expression vector or a vector expressing mutant QSLft(C-A), which has Ala substitutions at Cys89,90 to block N-formylglycine modification, establishing that QSLft functions as part of a two-state “catch or present” mechanism to regulate Wnt signaling, specifically to modulate the binding affinity of Wnts to HS chains on HSPGs to promote the HS-mediated presentation of Wnt ligand to its Frizzled receptor to initiate signaling.

**QSLft is a 6-O endosulfatase**

To define the substrate specificity of QSLft, we assayed radiolabeled disaccharide products generated by deaminative cleavage of [35S]GAG preparations (Shively and Conrad,
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1976; Rong et al., 2001) after incubation with wild type and (C-A) mutant forms of QSulf1 and with control cell extracts. Four major $^{35}$Sdisaccharide products were resolved by HPLC chromatography: GlcA-GlcNS6S, IdoA-GlcNS6S, IdoA2S-GlcNS, and IdoA2S-GlcNS6S (Table I). QSulf1 is preferentially active in the 6-O desulfation of IdoA2S-GlcNS6S units, with less pronounced effect on GlcA-GlcNS6S and no detectable effect on IdoA-GlcNS6S (Table I). In these assays, GlcA-GlcNS6S and IdoA2S-GlcNS6S are significantly decreased as a percentage of total radioactivity by $\sim$20 and $\sim$32%, respectively, with a parallel $\sim$50% increase of the reaction product IdoA2S-GlcNS. QSulf1 did not desulfate IdoA-GlcNS6S, even when enzyme reactions were performed for extended times. These findings show that QSulf1 is an HS-specific 6-O sulfatase with substrate specificity for a subset of 6-O–sulfated disaccharide substrates in HS. The disaccharide analyses also establish that QSulf1 is an endosulfatase, based on the quantitative extent of its activity on HS chains and its specificity for selected 6-O disaccharide substrates located at internal sites along HS chains. In contrast to the lysosomal GlcNR6sase, which functions exclusively as an exoenzyme on nonreducing-terminal GlcNR6S residues (Kresse et al., 1980), QSulf1 desulfates GlcNS6S residues located on the reducing side of GlcA units, hence functioning in an endolytic mode (Table I). Furthermore, QSulf1 does not desulfate the monosaccharide substrate N-acetylglucosamine 6-O sulfate, consistent with its activity as an endosulfatase, in contrast to the lysosomal exosulfatase GlcNR6Sase, which actively hydrolyzes this monosaccharide substrate both at pH 5.5 and 7.5 (Reissig et al., 1955; He et al., 1993) (Fig. 1 E). QSulf1 is also significantly more active in $^{35}$S$\text{SO}_4$ release from $^{35}$S[GAGs than is lysosomal GlcNR6Sase at both pH 5.5 and 7.5 (Fig. 1 F), further distinguishing their activities.

QSulf1 remodels the 6-O sulfation state of HS cell surface in vivo

To investigate whether QSulf1 is enzymatically active in vivo, we tested the effects of QSulf1 expression on 10E4 antibody immunoreactivity (IR) of cell surface HSPGs. 10E4 antibody specifically recognizes sulfated N-acetylglucosamine residues (David et al., 1992; Yip et al., 2002), and 10E4 IR to cell surface HSPGs is sensitive to chlorate treatment at concentrations that preferentially block 6-O sulfation state of HS cell surface in vivo...
tion (Safaiyan et al., 1999; Yip et al., 2002). 80% of 3T3 cells have cell surface 10E4 IR, as determined using a live cell staining assay, and chlorate treatment reduces 10E4 IR to <20% of cells (Fig. 2, A and B). Transfected QSulf1 also reduces 10E4 IR to a similar extent, whereas enzymatically inactive QSulf1(C-A) has no effect. Significantly, QSulf1 does not disrupt 10E4 IR on immediately adjacent cells (Fig. 2 A), even though QSulf1 is abundant on the surface of expressing cells. These observations indicate that QSulf1 functions cell autonomously to remodel the sulfation states of HSPGs on expressing cells. QSulf1 expression does not disrupt the sulfation state of CS on the cell surface, as assayed by live cell staining of QSulf1-transfected cells with a CS-specific antibody, CS56 (Avnur and Geiger, 1984), which recognizes sulfated epitopes also removed by chlorate treatment (Yip et al., 2002) (Fig. 2 C). These findings support the specificity of QSulf1 for HS, as observed in biochemical studies (Figs. 1, B and C).

QSulf1 also is an active 6-O endosulfatase in vivo with similar disaccharide substrate specificities as observed in biochemical assays (Table I; Fig. 3). [35S]GAGs were prepared from 293 cells that were stably transfected with wild-type and C-A mutant QSulf1 and metabolically labeled with [35S]SO4. Disaccharide analysis shows that labeled GlcA-GlcA-

Figure 2. QSulf1 desulfates cell surface HSPGs in living cells without affecting the stability of HSPG core proteins. (A) QSulf1 and chlorate desulfate cell surface HS, as assayed by IR to 10E4 antibody. Control 3T3 cells were transfected with empty vector, cultured with or without 25 mM chlorate to block sulfation, and then live cell stained with 10E4 antibody, and antibody reactivity was assayed by fluorescence microscopy. A majority of untransfected cells (Ctrl) express 10E4 IR on the cell surface, and chlorate treatment removes 10E4 IR. Cells transfected with QSulf1–MycHis or catalytic mutant QSulf1(C-A)–MycHis were live stained for extracellular 10E4 IR and then permeabilized to assay QSulf1 or QSulf1(C-A) with a His antibody. QSulf1-expressing cells (QSulf1) lose cell surface 10E4 IR, whereas QSulf1(C-A)-expressing cells remain immunoreactive, as shown in the overlay. Note that QSulf1 expression does not alter 10E4 IR on adjacent cells. An asterisk marks transfected cells and an arrow marks cells adjacent to QSulf1-expressing cells. (B) Quantitative analysis of the effects of QSulf1 expression and chlorate treatment on 10E4 IR. Cells stained with 10E4 were counted, and the percentage of 10E4 IR cells was calculated as the percent of total cells that were 10E4 stained in the control assay (Ctrl), or as the percent of transfected cells that expressed either QSulf1 or QSulf1(C-A). (C) QSulf1 expression does not alter the sulfation of cell surface CS. Extracellular CS was visualized with CS56 antibody in untransfected control (Ctrl) cultures treated with or without chlorate or in QSulf1-transfected cultures. An asterisk marks the cells transfected with QSulf1. Assays were conducted in duplicate in three independent experiments, counting >100 cells in each assay. (D) The protein core of Glypican1 remains on the cell surface of QSulf1-expressing cells and chlorate-treated cells. 3T3 cells cotransfected with Glypican1–Myc and untagged QSulf1 were live cell stained with Myc antibody to detect cell surface Glypican1–Myc. Cells were then permeabilized and immunostained for QSulf1 expression with QSulf1 antibody. Control cells were cotransfected with Glypican1–Myc and pAG empty vector plasmids, with or without chlorate treatment, followed by live cell staining to assay extracellular Glypican1–Myc. Similar Glypican1 staining was detected in control and QSulf1-transfected cells. (E) QSulf1 expression and chlorate treatment do not alter the stability or gel mobility of Glypican1. Western blot assays of cell extracts from 293 cells cotransfected with Glypican1–Myc, with pAG empty vector (Ctrl), QSulf1, or QSulf1(C-A) plasmids. Western blots were probed with anti-Myc and anti-QSulf1 antibodies.
The disaccharide components of 293T cells that expressed wild-type QSulf1 and enzymatically inactive QSulf1(C-A) mutant were prepared for disaccharide analysis. The radioactivity in each disaccharide product was quantified by scintillation counting. Results are presented as mol-

<table>
<thead>
<tr>
<th>Heparan sulfate</th>
<th>O-[35S]-sulfated disaccharides (% of total O-[35S]-sulfated disaccharides)</th>
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<tr>
<td>QSulf1 treatment</td>
<td>13.0 ± 1.0a</td>
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<tr>
<td>QSulf1(C-A) treatment</td>
<td>16.0 ± 2.0</td>
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<tr>
<td>Untreated control</td>
<td>19.0 ± 3.5</td>
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<tr>
<td>QSulf1-expressing cells</td>
<td>14.0 ± 1.0b</td>
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<tr>
<td>QSulf1(C-A)-expressing cells</td>
<td>16.3 ± 2.0</td>
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a P < 0.05 (t test).
b P < 0.1.

GlcNS6S and IdoA2S-GlcNS6S disaccharide units were quantitatively reduced in QSulf1-expressing cells compared with C-A mutant controls, and this decrease is accompanied by a parallel increase in IdoA2S-GlcNS. Disaccharide IdoA-

Figure 3. QSulf1 selectively removes 6-O sulfates from HS in vivo. [35S]HS prepared from metabolically labeled 293T cells was reacted with Myc bead–purified QSulf1 or catalytic mutant QSulf1(C-A). Untreated control was [35S]HS without treatment. To test whether QSulf1 functions in vivo, [35S]HS was prepared from stable 293T cell lines expressing QSulf1 or QSulf1(C-A) by metabolic labeling with [35S]SO4. [35S]-labeled disaccharides were generated by deaminative cleavage of HS, and reaction products were resolved by HPLC anion exchange chromatography. The radioactivity in each disaccharide product was quantified by scintillation counting. Results are presented as mol-

QSulf1 activity does not disrupt the accumulation of Glypican1 HSPG core protein, which is attached on the cell surface by a GPI linker. Immunological localization analysis shows that QSulf1-1 expressing 3T3 cells and chlorate-treated 3T3 cells have abundant levels of myc-tagged Glypican1 on these cell surfaces (Fig. 2 D). The electrophoretic mobility and levels of Glypican1 core protein also are unchanged in QSulf1-expressing cells, indicating that QSulf1 activity does not lead to processing or degradation of Glypican1 core proteins or its HS chains (Fig. 2 E). These findings support the conclusion that QSulf1 activity remodels the 6-O sulfation states of GlcA-GlcNS6S and IdoA2S-GlcNS6S disaccharide components of HSPGs, which are retained on the cell surface in their desulfated states.

Golgi-targeted QSulf1 is active in cell surface remodeling and Wnt signaling

During biosynthesis, QSulf1 transits through the ER and Golgi apparatus, where HSPGs are assembled (Prydz and Dalen, 2000), and transports together with HSPGs to the surface of expressing cells (Dhoot et al., 2001; Ohno et al., 2002; unpublished data). To investigate whether QSulf1 localization on the cell surface is required for its activity, we expressed Golgi-tethered (QSulf1-Golgi) and ER-tethered (QSulf1-ER) forms of QSulf1 in 3T3 cells and C2C12 myoblasts and then assayed for their biochemical and cell biological activities in remodeling the sulfation states of HS and for Wnt induction activities. Neither QSulf1-Golgi nor QSulf1-ER is detected on the cell surfaces of expressing cells (unpublished data), but they colocalize with Golgi and ER markers, respectively (Fig. 4 A). ER- and Golgi-tethered QSulf1 isolated from expressing cells are enzymatically active in biochemical assays for sulfate release using [35S]GAG substrates (Fig. 4 B). QSulf1-ER protein is inactive in remodeling 10E4 IR and has little or no activity in Wnt signaling, whereas the QSulf1-Golgi protein is fully active in remodeling 10E4 IR as well as inducing Wnt signaling (Figs.
These findings establish that QSulf1 plays an indirect role in promoting Wnt signal transduction by remodeling the 6-O sulfation states of extracellular HSPGs, either through its activity on the cell surface or its activity in the Golgi apparatus during HS biosynthesis.

To investigate the mechanisms by which QSulf1 6-O desulfation activity promotes Wnt signaling, we first tested the activity of soluble heparin and 6-O and 2-O chemically desulfated heparin on Wnt signal transduction in cells (Fig. 5 A). For these studies, Wnt1-expressing cells were cocultured with Wnt1-responsive C2C12 cells transfected with a TCF luciferase reporter to monitor Wnt signaling activity. At a concentration of 10 μg/ml, both soluble heparin and 2-O–desulfated heparin completely inhibit Wnt signaling activity, whereas 6-O–desulfated heparin has no inhibitory effect (Fig. 5 A). Therefore, soluble 6-O–sulfated HS could inhibit Wnt signaling by blocking the Frizzled receptor from binding the Wnt ligand. Alternatively, 6-O–sulfated HS could bind to Wnts and prevent Wnt presentation to its Frizzled receptor. To investigate whether HS blocks Frizzled receptor activity, we compared the Wnt1 signaling activity in wild-type CHO cells that express HSPGs and in pgd677 mutant CHO cells that are defective in HS biosynthesis (Bai et al., 1999). Wnt1 signaling in CHO cells is dependent on expression of a transfected Frizzled 3 (mFZ3) receptor, which increases the response of these cells to Wnt1 by 12–15-fold in a coculture system with Wnt1-expressing cells, as assayed using a LEF/TCF luciferase reporter (unpublished data). CHO cells are dependent on transfected mFZ3 receptor for Wnt1 signal transduction, which makes it possible to directly monitor the requirement for
HS in the response of Frizzled receptor to Wnt signal. In these mFZ3-dependent Wnt signaling assays, wild-type and pgsd677 mutant CHO cells were found to be inactive in Wnt1 signal transduction in the absence of Wnt1 ligand (Fig. 5 B), whereas in response to Wnt1 ligand, mFZ3-expressing wild-type CHO cells more actively respond to Wnt1 than do HS-deficient pgsd677 cells (which have only a slightly elevated response to Wnt1 compared with unstimulated control cells) (Fig. 5 B). These findings show that HS does not act as a general repressor of ligand-independent mFZ3 receptor activity. On the contrary, they provide evidence that HS is required for efficient mFZ3 receptor activation in response to Wnt1 ligand. Therefore, soluble sulfated heparin inhibits Wnt signaling probably by binding to Wnt ligand and subsequently preventing the release of the Wnt ligand to the receptor.

To investigate whether 6-O desulfation of heparin affects Wnt binding, we developed a competition assay with heparin–agarose beads to compare the binding affinity of XWnt8 to heparin or 6-O–desulfated heparin. XWnt8 tagged with HA is soluble and biologically active in the canonical Wnt signaling pathway (Hsieh et al., 1999; Piccolo et al., 1999). In this assay, HA–XWnt8 binds with high affinity to heparin–agarose beads (unpublished data), consistent with earlier studies of Wing binding to HS (Reichsman et al., 1996). We then tested the ability of increasing concentrations of heparin, pretreated either with Qsulf1 or inactive Qsulf1 (C-A) mutant protein, to release bound HA–XWnt8 from heparin–agarose beads, as assayed by Western blot analysis of HA–XWnt8 released into the supernatant fraction (Fig. 5 C). We found that heparin treated with inactive Qsulf1 (C-A) promoted the concentration-dependent release of HA–XWnt8 from heparin beads; by contrast, Qsulf1-treated heparin released only small amounts of HA–XWnt8, and this release was not concentration dependent, reflecting low affinity binding of HA–XWnt8 to 6-O–desulfated heparin (Fig. 5 C).

The activity of Qsulf1 in Wnt binding to HS was also tested in XWnt8 binding to Glypican1, a cell surface HSPG that mediates Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). A soluble form of Glypican1 tagged with alkaline phosphatase (AP) was used in the binding assay to allow Glypican1 purification from the media of transfected cells (Chen and Lander, 2001). HA-tagged XWnt8 was incubated with the soluble Glypican1–AP, and complexes formed were immuno-
precipitated by agarose beads coupled with monoclonal AP antibodies, followed by probing Western blots of protein complexes with HA-specific antibodies (Fig. 5D). Pretreatment of Glypican1 with heparinase completely blocked its binding to XWnt8, establishing that XWnt8 binding to Glypican1 is mediated through the HS chains and not the protein core. QSulf1 treatment of Glypican1–AP significantly diminished, but did not completely block, formation of HA–XWnt8 binding, compared with treatment with C-A mutant QSulf1, which did not diminish complex formation (Fig. 5D). QSulf1 treatment also blocked the binding of Glypican1–AP to XWnt8 expressed on the surface of living transfected 293T cells, as detected by immunohistochemical detection in a cell binding assay (Hsieh et al., 1999; Wu and Nusse, 2002), whereas QSulf1(C-A) treatment did not prevent binding (Fig. 5E). Control and C-A mutant QSulf1–treated Glypican1–AP abundantly bound to the surfaces of HA–XWnt8–expressing cells, as detected by double immunostaining with HA antibodies (unpublished data). Together, these Wnt binding studies reveal that QSulf1 6-O sulfatase activity reduces the binding of Wnt to HS on Glypican1. The results of these binding studies are consistent with a two-state catch or presentation model (Fig. 6) in which 6-O–sulfated HS on cell surface HSPGs binds Wnts in a high affinity state to “catch” Wnt ligands and compete with the binding of Wnts to Frizzled receptor (Fig. 6A). The HS 6-O desulfatation activity of QSulf1 would convert the cell surface HS to a low affinity binding state for Wnts, which allows their HS-dependent “presentation” to the Frizzled receptors to initiate Wnt signal transduction (Fig. 6B). By this mechanism, QSulf1, which is expressed in localized populations of somite and neural progenitor cells, would pattern Wnt signaling responses to localized populations of progenitors responding to widely dispersed Wnt signals.

Figure 6. A two-state catch or presentation model of QSulf1 regulation of Wnt signaling. (A) In QSulf1–nonexpressing embryonic cells, HS chains on cell surface HSPGs are in a 6-O–sulfated state, which binds with high affinities to catch Wnt ligands, preventing functional interactions of bound Wnts with their Frizzled receptors. (B) In QSulf1–expressing cells, selective 6-O desulfation activity of QSulf1 removes 6-O sulfates from HS chains on cell surface HSPGs to convert HS to a low affinity binding state for Wnts. 6-O–desulfated HS then can present Wnt ligands to Frizzled receptor and can form functionally active Wnt–HS–Frizzled receptor complexes for initiation of Wnt signal transduction.

Discussion

Our biochemical and cell expression studies identify QSulf1 as an HS-specific 6-O endosulfatase with substrate specificity for disulfated GlcA–GlcNS6S and trisulfated IdoA2S–GlcNS6S disaccharide components of cellular HS chains. QSulf1 does not appear to desulfate -IdoA-GlcNS6S- sequences, providing evidence for functional selectivity of QSulf1 activities. Also, QSulf1, both in vitro and in vivo, desulfates only a small fraction of GlcA-GlcNS6S disaccharide residues, suggesting that QSulf1 is active with only a subset of GlcA-GlcNS6S residues in HS chains. Compared with its low activity on GlcA-GlcNS6S residues, QSulf1 induces appreciable 6-O desulfation of trisulfated IdoA2S–GlcNS6S units, which are major sulfated constituents of cellular HS chains. These findings are consistent with immunochemical studies with 10E4 antibody in which we show that QSulf1 expression remodels the 10E4 IR on the surface of expressing cells. HSulf1 and HSulf2, human orthologues of QSulf1, also desulfate -IdoA2S-GlcNS6S- units on intestinal heparin (Morimoto-Tomita et al., 2002). Heparin is enriched in IdoA and O-sulfation, in contrast to cell-derived HS, which contains more GlcA and has less 2-O–sulfated sugars (Vives et al., 1999), which accounts for our identification of GlcA-GlcNS6S as a QSulf1 substrate. The substrate specificity and high level activity of QSulf1 on cell-derived HS establish QSulf1 as a unique 6-O endosulfatase that removes 6-O sulfates from sulfated residues located within HS chains, in contrast to lysosomal GlcNR6Sase, which is an exosulfatase involved in the sequential degradation of HS chains (Kresse et al., 1980).

QSulf1 6-O endosulfatase activity for disulfated GlcA-GlcNS6S and trisulfated IdoA2S–GlcNS6S disaccharide components of cellular HS chains attenuates the binding affinity of XWnt8 ligand to HS chains on Glypican1, which plays a central role in developmental signaling (Lin and Perri-
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mon, 1999; Tsuda et al., 1999; Baeg et al., 2001; Nybakken and Perrimon, 2002), leading to stimulation of Wnt signaling. Although the specific structural interactions between Wnt ligand and HS residues are not yet known, it is notable that the trisulfated IdoA2S-GlcNS6S disacharide contributes directly to the high affinity binding of bFGF to HS, and the 6-O sulfate of this trisulfated disacharide is required for FGF-dependent receptor dimerization and activation (Schlessinger et al., 2000; Pye et al., 2000). These observations predict that QSulf1 activity will block HS-dependent FGF signaling, in contrast to Wnt signaling, which is stimulated by QSulf1 (Dhoot et al., 2001). QSulf1, therefore, may have a dual developmental function as a positive regulator of Wnt signaling and a negative regulator of FGF signaling.

Our findings provide evidence that QSulf1 plays a positive role in the regulation of Wnt signaling. QSulf1 is tethered to the cell surface and cell autonomously remodels the 6-O sulfate of extracellular HSPGs of Wnt-responsive somite and neural progenitors in embryos (Dhoot et al., 2001). Notum is another HSPG-modifying enzyme that regulates Wnt signaling in embryos (Giraldez et al., 2002). However, in contrast to QSulf1, Notum is released from Wg-producing cells to establish a morphogen gradient of Wg ligand presumably through its activity to degrade HS on HSPGs. Drosophila Notum mutants show much expanded Wg distribution and enhanced Wg activity in embryonic patterning, suggesting that Notum negatively regulates Wg signaling in Wg-responsive cells. By contrast, QSulf1 positively regulates Wnt signaling when localized either on the cell surface or targeted to the Golgi apparatus by actively remodeling the 6-O sulfate concentrations of HS chains, which does not degrade HS or promote the turnover of HSPG core protein.

Our study of QSulf1 activity on Wnt binding to HS sheds light on the mechanism of QSulf1 function in Wnt signaling. We show that soluble 6-O–sulfated heparin binds to Wnt ligand with high affinity and strongly inhibits Wnt signaling. QSulf1 significantly reduces, but does not eliminate, the binding of XWnt8 to HS through its 6-O desulfotase activity to promote Wnt signaling. Selective 6-O desulfation by QSulf1 does not remove all sulfates from HS. The residual sulfated residues on QSulf1-desulfated HS are essential for HS-mediated transduction of Wnt signals and Frizzled activation, based on previous findings that complete loss of HS sulfate in sulfateless mutant Drosophila or by chlorate treatment blocks Wnt signaling (Reichsman et al., 1996; Lin and Perrimon, 1999; Dhoot et al., 2001; Toyoda et al., 2000). CHO cells deficient in HS biosynthesis are also defective in Frizzled-dependent Wnt signaling, further establishing that HS is required for Frizzled function. Our findings also reveal that QSulf1 targeted to the cell surface or the Golgi apparatus actively remodels the sulfate states of cell surface HSPGs and promotes Wnt signaling. Therefore, QSulf1 itself does not function dynamically in the presentation of Wnt ligands to Frizzled receptors, but rather selectively removes 6-O sulfates from HS on the cell surface to convert the HS from a high affinity binding state to a low affinity binding state to allow the formation of functional Wnt–Frizzled complexes. Together, these findings support a static two-state catch or presentation model for QSulf1 regulation of Wnt signaling (Fig. 6). According to this model, embryonic cells that do not express QSulf1 would have sulfated HSPGs on the cell surface in a high affinity state that catch Wnts and compete for binding of Wnts to their Frizzled receptors. QSulf1 expression in localized populations of embryonic progenitors would reduce 6-O HS sulfation to lower the binding affinity between Wnts and HSPGs, and 6-O–desulfated HS in the low affinity binding state would present Wnt ligand to promote the formation of Wnt–Frizzled complexes to initiate Wnt signaling. In this regard, it is interesting to note that Frizzled-related proteins, such as sFRP-1, have heparin-binding domains that promote the formation of sFRP-1 and Wg complexes (Chong et al., 2002), suggesting that Wnts interacting with QSulf1-desulfated HS chains form a ternary complex with the Frizzled receptor, similar to the FGF–HS–FGFR complex that is required to initiate FGF signaling (Schlessinger et al., 2000). Therefore, the presentation of Wnt ligand by 6-O–desulfated HS in the low affinity binding state would provide a static mechanism to promote Wnt–Frizzled interaction in QSulf1-expressing cells. However, we also show that soluble heparin blocks Wnt signaling, suggesting that trisulfated HS may immobilize Wnt ligand on the cell surface, and QSulf1 activity reduces Wnt binding affinity of HS to enhance the access of Wnt ligand to Frizzled receptor, as shown by biochemical binding assays. We also show that chemically 6-O–desulfated soluble heparin does not promote Wnt signaling, suggesting that the residual 6-O sulfate groups on QSulf1-desulfated HS may be involved in Wnt binding to its Frizzled receptor. Structural studies of the interactions of QSulf1-modified HS substrates with Wnt ligands and their Frizzled receptors will provide insights into the specific mechanisms by which QSulf1 activity regulates Wnt signaling and will provide a basis for the development of therapeutic reagents to attenuate pathological HSPG-dependent signaling responses through control of angiogenesis and cell migration in diseases such as cancer (Sasaki et al., 1999; Lundin et al., 2000; Selva and Perrimon, 2001).

Materials and methods

Expression plasmids

QSulf1 and Glypican1 were cloned in pAG expression vector with a Myc-His COOH-terminal tag. The expression vector for a soluble form of Glypican1–AP lacking GPI linkage groups was provided by A. Lander (University of California, Irvine, CA) (Chen and Lander, 2001). HA–XWnt8 was cloned into pCS2 expression vector (Piccolo et al., 1999). QSulf1–ER expression vector was constructed by replacing the COOH-terminal Myc-His tag with a KDEL coding sequence (Munro and Pelham, 1987). QSulf1–Golgi expression plasmid was constructed by replacing the NH2-terminal signal peptide of QSulf1 (amino acids 1–21) with NH2-terminal amino acids 1–81 of the human β 1,4-galactosyltransferase (Gleeson et al., 1994). pECFP-Golg1 and pECFP-ER were used as transfection markers to assay Golgi apparatus and ER expression (CLONTECH Laboratories, Inc.).

Cell culture and DNA transfection

All cell lines were cultured in DMEM with 10% FBS (Invitrogen). Cells were transfected with Fugene 6 (Roche) in 24-well plates for immunohistochemical analysis and Wnt1 assays, as described previously (Dhoot et al., 2001). QSulf1, Glypican1, Glypican1–AP, and XWnt8 proteins were expressed in 293T cells cultured in 100-mm dishes using a standard lipofectamine transfection protocol (Invitrogen). Stable QSulf1-expressing 293T cell lines were generated by selecting transfected 293T cells with 250 μg/ml hygromycin (Sigma-Aldrich) 48 h after transfection. Expressed proteins, including QSulf1, Glypican1, and HA–XWnt8, were extracted from cells 24 h after transfection. To produce Glypican1–AP, transfected cells were
switched to serum-free DMEM/F12 (Invitrogen) 24 h after transfection, and conditioned medium was collected 16 h later. Protein expression was monitored by Western blot electrophoresis, followed by enzymatic or binding assays, as described below. As noted, chlorate-treated cultures were incubated with 25 mM chlorate for 18 h before being processed for immunocytochemistry and Western blot analysis.

Preparation of $^{35}$S-GAG substrates
Confluent 293T cells cultured in 100-mm dishes, either untransfected or stable lines expressing QSUf1 or control proteins, were transferred to low-sulfate F12 medium with 1% FBS and metabolically labeled for 5 h in 100 

Microcellular mass digestion products were removed from heparinase- and chondroitinase-treated $^{35}$S-GAG preparations by centrifugation in 5-kD filter units. To differentially deplete $^{35}$S-GAGs of HS or CS, samples were digested for 4 h at 37°C with either heparinase I and II (0.1 U/ml; Sigma-Aldrich) or chondroitinase ABC (0.1 U/ml; Sigma-Aldrich), followed by heat inactivation. Prior to enzyme assays, low molecular mass digestion products were removed from heparanase- and chondroitinase-treated $^{35}$S-GAG preparations by centrifugation in 5-kD filter units.

QSUf1 enzyme assays
293T cells transfected with Myc-tagged QSUf1 and control plasmids were lysed in 500 

To produce HA–XWnt8, 293T cell cultures transfected with HA–XWnt8 expression plasmid were lysed in 500 

Binding assay for HA–XWnt8- or HA–XWnt8-expressing cells
To produce HA–XWnt8, 293T cell cultures transfected with HA–XWnt8 expression plasmid were lysed in 500 

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References
Bai, X., G. Wei, A. Sinha, and J.D. Esko. 1999. Chinese hamster ovary cell mutants expressing Glypicanc1–AP were treated with QSUf1 or control overnight. For binding to HA–XWnt8, 50 

Competitive assay for HA–XWnt8 binding to heparin
Heparin immobilized on 100 

Structural analysis of $^{35}$S-labeled HS
HS chains were cleaved at N-sulfated glucosamine residues by treatment with nitrous acid at pH 1.5 (Shively and Conrad, 1976), followed by reduction with NaBH$_4$. Labeled deamination products were fractionated by gel chromatography on a column (1 × 180 cm) of Sephadex G-25 eluted with 0.2 M NaH$_2$PO$_4$. Samples of isolated disaccharides were analyzed on a Partisil-10 SAX column eluted at a rate of 1 ml/min with stepwise increasing concentrations of KH$_2$PO$_4$ (Rong, et al., 2001).

Immunocytochemistry and Western blots
Cells for immunocytochemical analysis were cultured on acid-treated coverslips. To assay cell surface markers, cells were live cell stained by incubating cells with the primary antibody in culture medium for 2 h, rinsed with PBS, and then permeabilized by fixation with 4% paraformaldehyde/PBS at room temperature for 15 min for immunostaining to identify QSUf1–Myc-His-expressing cells. After washing, cells were incubated for 2 h with the secondary antibody or double stained in antibody dilution buffer (0.1% Triton X-100 in PBS, 20% goat serum). Cells were subsequently washed with PBS 3 times and mounted and analyzed by fluorescence microscopy. Western blots were prepared as described previously (Dhout, et al., 2001). The primary antibodies used included mouse anti-Myc (9E10; 1:300 for immunocytochemistry and 1:2,000 for Western blots), rabbit anti-His (1:200; Santa Cruz Biotechnology, Inc.), mouse anti-HS (10E4; 1:150; Seikagaku), mouse anti-CS (CS6; 1:100; Sigma-Aldrich), rabbit anti–QSulf1 hydrophilic domain (1:200 for immunostaining and 1:2,000 for Western blotting; Calico), mouse anti-TGN 38 (Ab-1; 1:1,000; Oncogene); mouse anti-PDI (1:100; Affinity BioReagents, Inc.), mouse anti–alkaline phosphatase (8B6; 1:4,000; Sigma-Aldrich), and mouse anti–TGN 38 (Ab-1; 1:1,000; Calbio). Proteinase K was then heat inactivated, and $^{35}$S-GAGs were precipitated in three volumes of 95% ethanol, 1 

To generate soluble Glypicanc1–AP, conditioned medium collected from transfected cells was concentrated 10-fold through 10-kD Centriprep (Amicon) columns, and the amount of concentrated proteins was quantified using a colorometric dye concentrate reagent (Bio-Rad Laboratories). The concentrated proteins containing Glypicanc1–AP were treated with QSUf1 or control overnight. For binding to HA–XWnt8, 50 

Preparation of [35S]GAG substrates
[35S]GAGs were reconstituted in water at a concentration of 10$^7$ cpm/ml for use as substrates in QSulf1 enzyme assays or digestion with proteinase K (10 

Preparation of [35S]GAG substrates
$[35S]$GAGs were reconstituted in water at a concentration of $10^7$ cpm/ml for use as substrates in QSulf1 enzyme assays or directly for disaccharide assays. To differentially deplete $^{35}$S-GAGs of HS or CS, samples were digested for 4 h at 37°C with either heparinase I and II (0.1 U/ml; Sigma-Aldrich) or chondroitinase ABC (0.1 U/ml; Sigma-Aldrich), followed by heat inactivation. Prior to enzyme assays, low molecular mass digestion products were removed from heparanase- and chondroitinase-treated $^{35}$S-GAG preparations by centrifugation in 5-kD filter units.

QSUf1 enzyme assays
293T cell cultures transfected with Myc-tagged QSUf1 and control plasmids were lysed in 500 

Heparinase- and chondroitinase-treated $^{35}$S-GAG preparations were digested in 10$^7$ cpm/ml for use as substrates in QSulf1 enzyme assays or digestion with proteinase K (10 

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