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Cooperation of binding sites at the hydrophilic domain of cell-surface sulfatase Sulf1 allows for dynamic interaction of the enzyme with its substrate heparan sulfate

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ABSTRACT

Background: Sulf1 is a cell-surface sulfatase removing internal 6-*O*-sulfate groups from heparan sulfate (HS) chains. Thereby it modulates the activity of HS-dependent growth factors. For HS interaction Sulf1 employs a unique hydrophilic domain (HD).
Methods: Affinity-chromatography, AFM-single-molecule force spectroscopy (SMFS) and immunofluorescence on living cells were used to analyze specificity, kinetics and structural basis of this interaction.
Results: Full-length Sulf1 interacts broadly with sulfated glycosaminoglycans (GAGs) showing, however, higher affinity toward HS and heparin than toward chondroitin sulfate or dermatan sulfate. Strong interaction depends on the presence of Sulf1-substrate groups, as Sulf1 bound significantly weaker to HS after enzymatic 6-*O*-desulfation by Sulf1 pretreatment, hence suggesting autoregulation of Sulf1/substrate association. In contrast, HD alone exhibited outstanding specificity toward HS and did not interact with chondroitin sulfate, dermatan sulfate or 6-*O*-desulfated HS. Dynamic SMFS revealed an off-rate of 0.04/s, i.e., ~500-fold higher than determined by surface plasmon resonance. SMFS allowed resolving the dynamics of single dissociation events in each force–distance curve. HD subdomain constructs revealed heparin interaction sites in the inner and C-terminal regions of HD.
Conclusions: Specific substrate binding of Sulf1 is mediated by HD and involves at least two separate HS-binding sites. Surface plasmon resonance K_D-values reflect a high avidity resulting from multivalent HD/heparin interaction. While this ensures stable cell–surface HS association, the dynamic cooperation of binding sites at HD and also the catalytic domain enables processive action of Sulf1 along or across HS chains.
General significance: HD confers a novel and highly dynamic mode of protein interaction with HS.

1. Introduction

Mammalian embryogenesis is characterized by highly ordered cell differentiation and proliferation. These processes are triggered and regulated by growth factors and morphogens like those of the fibroblast growth factor (FGF) family, Wnts and many others, which form active signaling complexes with their cognate receptors at the cell surface. The glycosaminoglycan (GAG) heparan sulfate (HS) is of central importance for a multitude of these interactions by either acting as coreceptor or regulating distribution and bioavailability of signaling molecules. HS is built as a long, linear chain of up to 150 repetitively ordered disaccharides containing glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) and is synthesized on a linker saccharide that is attached to a membrane-anchored core protein. During biosynthesis GlcA can be epimerized to iduronic acid (IdoA) and by the action of *N*-deacetylases/sulfotransferases regions with a high or intermediate degree in *N*-sulfation are established, although also regions with no modification remain. Particularly the regions with high *N*-sulfation can be further sulfated at the 2-*O*-position of IdoA/GlcA as well as at the 6-*O*- and rarely 3-*O*-position of the glucosamine leading to discrete sulfation patterns with highly sulfated S-domains, which are the hotspots of growth factor binding [1–3].

Many growth factor interactions with HS are dependent on 6-*O*-sulfate groups (6*S*-groups) in the highly sulfated S-domains of HS [4–7]. Moreover, two cell surface located sulfatases, Sulf1 and Sulf2,

Abbreviations: AFM, atomic force microscopy; CS, chondroitin sulfate; DMMB, 1,9-dimethylmethylene blue; DS, dermatan sulfate; DSMFS, dynamic SMFS; ECM, extracellular matrix; FGF, fibroblast growth factor; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfoglucosamine; GST, glutathione *S*-transferase; HD, hydrophilic domain; HRP, horseradish peroxidase; HS (HS + 6S), heparan sulfate; HS − 6S, 6-*O*-desulfated HS; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; K5_NS, *N*-sulfated K5-polysaccharide; MBP, maltose binding protein; NS, *N*-sulfate; 2*S*, 2*O*-sulfate; 6*S*, 6*O*-sulfate; SAX-HPLC, strong anion exchange-high performance liquid chromatography; SDS, sodium dodecyl sulfate; SMFS, single molecule-force spectroscopy; SPR, surface plasmon resonance; UA, hexuronic acid

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were discovered showing enzymatic activity directed against 6*S*-groups, thereby introducing a unique tool to post-synthetically edit established HS sulfation patterns [8,9]. As a consequence, HS interactions with ligands like FGFs, Wnts and others are modulated [10–14]; in fact severe developmental malformations were observed in Sulf1/Sulf2 knock-out mice [7,12,15–18]. At the molecular level, the Sulfs exhibit a predominant specificity toward 6*S*-groups of the trisulfated disaccharide ΔUA(2S)-GlcNS(6S), but also toward ΔUA-GlcNS(6S) [9–11,19]; both species are associated to S-domains.

Sulf dysregulation is frequently observed in primary tumors and affected signaling pathways are under intensive research [20–22]. In order to develop tumor therapies, not only inhibition of the Sulfs using glucosamine-6-*O*-sulfamate derivatives or heparin mimetics was analyzed [23,24], but also activating Sulf1 as a tumor suppressor [25,26] has been considered. Thus, it is of major importance to understand Sulf interaction with HS or other GAGs in more detail. As Sulf1 and Sulf2 are the only known sulfatases present on the cell surface, the focus was put on the hydrophilic domain (HD), which is decisive for HS interaction and exclusively found in Sulf1 and Sulf2. HD is highly conserved between isoforms and species except for a less conserved inner region [9]. Using deletion mutants of Sulf1 as well as isolated HD it was shown that HD indeed is needed for enzymatic turnover of HS and responsible for cell surface localization of Sulf1 and Sulf2 through heparinase-sensitive interactions [27–29]. Accordingly, a high affinity of HD toward heparin and HS could be demonstrated *in vitro* by affinity chromatography and by surface plasmon resonance (SPR) yielding K_D-values in the low nanomolar range [29]. While other groups reported a rather unspecific interaction of HD from quail Sulf2 with HS and CS/DS [27], a strict dependence of the HS interaction on the presence of 6*S*-groups could be confirmed for HD from human Sulf1 [29].

On average, each fourth of the 319-amino acid residues HD is basic and could potentially be part of an HS interaction site. This is quite uncommon for HS interacting proteins, many of which contain so-called Cardin/Weintraub motifs that are more discrete and significantly smaller [30–32]. In the present study, we identified structural elements that are responsible for interactions on both the protein and the GAG side. For the first time full-length Sulf1 protein was subjected to interaction analyses *in vitro* and shown to differentially bind to sulfated GAGs. We demonstrate that the highly specific binding to Sulf1-substrate groups on HS or heparin is mediated by HD. Determination of off-rates by AFM-dynamic-force-spectroscopy (DSMFS) for the HD/heparin interaction revealed significant and interesting differences to off-rates determined previously by SPR. These differences are explained by multivalent interactions, which could be resolved by DSMFS only.

2. Materials and methods

2.1. Construction of expression plasmids

For heterologous expression of the isolated HD of Sulf1 in N-terminal fusion with maltose binding protein (MBP) in *E. coli*, cDNA fragments encoding HD (residues K^{417}–K^{735}), HD_I (residues K^{417}–G^{458}), HD_II (residues K^{417}–H^{616}), HD_III (residues Q^{459}–H^{616}), HD_IV (residues Q^{459}–K^{735}) and HD_V (residues K^{617}–K^{735}) were amplified by PCR, using pKM263-HD without Strep tag II [29] as a template, and cloned via AvaI/BamHI restriction sites into the pMAL-c5X vector (New England Biolabs). To the HD-encoding sequences an N-terminal *PreScission Protease*-cleavable coding sequence and a C-terminal Strep tag II as well as the restriction sites were added (for primer sequences see Supplemental Table 1).

2.2. Generation of GAG affinity columns

The heparan sulfate-affinity chromatography columns generated by covalently linking HS, pretreated or not with Sulf1ΔHDC, to 1 mL HiTrap NHS-activated columns (GE Healthcare) are described in ref. [29]. Accordingly, columns containing chondroitin sulfate A (Sigma-Aldrich), chondroitin sulfate B/dermatan sulfate (DS) (Sigma-Aldrich) and heparin (Sigma-Aldrich) were generated. All the different GAGs had been immobilized in sufficient amounts as could be proven by flushing the columns with solutions of the sulfated GAG-specific dye dimethylmethylene Blue (DMMB) and recording the retention volumes needed to detect the dye in the eluate (Fig. S1A and B). Although DMMB binding capacities largely differed between the columns and a direct comparison of the immobilization degree is difficult due to GAG-type specific binding of DMMB [33], the amounts of immobilized GAG were sufficient to detect bound HD by Western blotting in the eluate of any column. To control for unspecific interactions with the matrix a column reacted with ethanolamine was generated.

2.3. GAG interaction analyses with HD constructs

MBP–HD constructs were expressed in *E. coli*. Pelleted cells were lysed and cleared supernatants loaded on amylose-affinity columns using a chromatography system and PBS pH 7.3 as running buffer. After washing bound proteins were eluted by a step to 100% elution buffer (PBS pH 7.3, 0.01 M maltose) over 5 CV. Following purification via amylose-affinity chromatography, equimolar concentrations of MBP fusion proteins were subjected to cleavage by *PreScission Protease* (GE Healthcare). Reaction mixtures were cleared by centrifugation and supernatants loaded on the GAG columns using a chromatography system and 0.02 M Tris pH 7.4 as binding buffer. After washing with binding buffer bound proteins were gradually eluted in a linear gradient over 10 CV from 0% to 100% elution buffer (0.02 M Tris pH 7.4, 1.5 M NaCl). A 5 CV delay was enclosed to ensure 100% elution buffer conditions. Chromatography runs were monitored by measuring UV-absorbance (at 280 nm) and conductivity. The latter allowed for calculating NaCl concentrations at UV-peak positions or in collected fractions. One milliliter fractions were taken during each, loading, washing and elution steps. Samples, representative for flow-through and washing, as well as elution fractions E1–E10 were separated by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF)-membrane (Carl Roth) for detection with either an anti-MBP serum (NEB) or HRP-conjugated StrepTactin (IBA). To control for possible effects of cleaved MBP on GAG interaction, purified MBP–HD was subjected to *PreScission Protease* cleavage during dialysis against binding buffer, loaded thereafter on an amylose matrix to remove cleaved MBP and then subjected to GAG affinity chromatography.

2.4. GAG interaction analyses with Sulf1

Proteins in conditioned medium of stably Sulf1CA-RGSH_6-expressing cells [11] were precipitated with 50% (w/v) $(NH_4)_2SO_4$. Following centrifugation, pellets were resuspended in and dialyzed against Ni^{2+}-affinity chromatography binding buffer (0.02 M Tris pH 7.4, 0.5 M NaCl, 0.04 M imidazole). Cleared dialysates were loaded on a HisTrap HP 1 mL Ni^{2+}-affinity column (GE Healthcare) using a chromatography system. After washing with 20 CV binding buffer, bound proteins were gradually eluted in a linear gradient over 15 CV from 0% to 100% elution buffer (0.02 M Tris pH 7.4, 0.05 M NaCl, 0.5 M imidazole). Elution fractions containing Sulf1CA, as verified by Western blotting, were pooled and loaded on the GAG columns as described above for the HD constructs. Analysis of chromatography fractions was carried out accordingly. The same procedure was used for GAG interaction analysis of Sulf1ΔHD-RGSH_6 [29]. For Western blotting anti-RGS-His_6 antibodies (Qiagen) or a Sulf1 specific antiserum (kindly provided by Shire Human Genetic Therapies, Inc.) were used.

2.5. Immunofluorescence microscopy

The cell surface localization of MBP–HD was analyzed by immunofluorescence microscopy. Untransfected HT1080 cells were cultivated on poly-L-lysine coated cover slips to a confluence of 70%. Binding of