PROGRESS REPORT for Mizutani Foundation Research Grant

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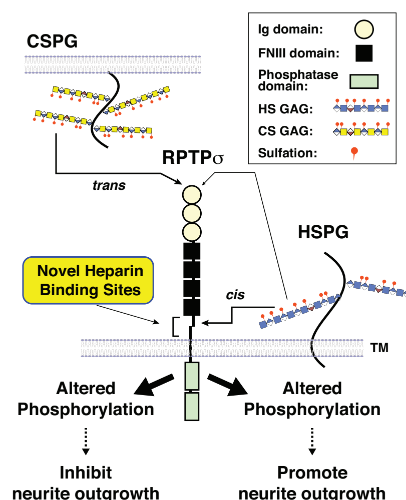
Abstract

Emerging evidence suggests that proteoglycans are independent signaling molecules, binding directly to transmembrane receptors to exert distinct biological effects. In the nervous system, the glycosaminoglycan (GAG) chains of proteoglycans bind to members of the Type IIa receptor protein tyrosine phosphatases (RPTPσ/RPTPδ/LAR). RPTPσ is composed of two cytoplasmic phosphatase domains and extracellular immunoglobulin (Ig) domains followed by fibronectin type III (FNIII) repeats (Figure). Previous mutagenesis and structural studies have shown that a GAG-binding site for both heparan sulfate (HS) and chondroitin sulfate (CS) lies in the first Ig domain of RPTPσ and comprises an extended positively charged surface of basic residues (Lys- and Arg-loop). This has led to the conundrum that both HS and CS bind to the same site but produce opposite actions: HS promotes axonal growth and CS impedes the growth of axons.

We have investigated whether the complex formation between full length RPTPσ and GAGs impacts the receptor's enzymatic activity under physiological conditions. When 293 cells expressing wild-type RPTPσ were treated with heparin, a mimic of HS, the level of Tyr phosphorylation (pTyr) was transiently upregulated. Intriguingly, the increase in pTyr by heparin was found even after disruption of the Lys/Arg loop (ΔLys mutation) whereas CS-E-induced upregulation was substantially reduced by the mutation. Solid-phase binding assays with the extracellular domain (ECD) of RPTPσ showed the presence of multiple binding sites for heparin on RPTPσ. The fact that heparin binding upregulated pTyr independently of Lys/Arg loop binding site on RPTPσ led us to identify novel heparin binding sites on the receptor.

To further characterize the novel binding site(s), a "heparin protection assay" was performed to identify potential amino acids, followed by mutagenesis of these amino acids together with heparin affinity chromatography. This allowed the identification of 2 novel heparin-binding sites located in the juxtamembrane domain (Figure). Disruption of all 3 heparin-binding sites (one in the 1st Ig domain and two in the juxtamembrane domain) completely eliminated the binding to heparin.



One question that arises here is whether these novel heparin-binding sites are involved in the altered phosphorylation upon heparin binding. When the heparin-binding deficient mutant was expressed on 293 cells, we observed very high level of baseline

pTyr without heparin. Addition of heparin did not alter the level of phosphorylation. This implies that elimination of all heparin binding sites on RPTPσ caused the dysregulation of pTyr within the cells. Thus, we propose a model for differential interactions of HS and CS GAG chains with RPTPσ (Figure). HSPGs on the same cell surface interact in *cis* with RPTPσ through the novel binding sites and regulate the enzymatic activity of RPTPσ. When CSPGs are presented in *trans*, the binding is through the first Ig domain and triggers the changes in phosphorylation of distinct sets of proteins within the cells, resulting in the recognition of CS as inhibitory cues by RPTPσ. Our discovery of new heparin binding sites near the transmembrane domain of RP provides new insights into GAG signaling.

Objectives:

Proteoglycans in the extracellular matrix play central roles in maintaining tissue architecture and modulating cell signaling. Emerging evidence suggests that proteoglycans themselves may be independent signaling molecules, binding directly to transmembrane receptors to exert distinct biological effects. Most of these interactions depend on binding of proteins to the negatively charged glycosaminoglycan (GAG) chains of the proteoglycans. GAGs are the most abundant heteropolysaccharides in the body and their specific GAGs of physiological significance are heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate, and hyaluronic acid. Of biological importance in the central nervous system (CNS) are chondroitin sulfate proteoglycan (CSPG) and heparan sulfate proteoglycan (HSPG).

We have identified Reticulon 4 receptor (Nogo-66 Receptor) as one of long-sought CSPG receptors[1]. RPTPσ and RPTP-LAR, members of type IIa receptor protein tyrosine phosphatase subfamily, have also been classified as CSPG receptors[2,3]. The question arises as to how RPTPσ can mediate both growth promotion and growth inhibition by interaction with HS and CS. One solution was proposed by Coles et al.[4], where HS and CS exert opposite effects on the oligomerization of RPTPσ, because binding to HS, but not to CS, induces clustering of the extracellular region of RPTPσ. However, we found that both HS and CS-E induced the clustering of RPTPσ. Thus, we hypothesized that another mechanism exists to mediate biologically opposite signals from HS and CS through the same receptor.

***Aim 1*: To identify a novel heparin binding site on RPTPσ**

Hypothesis: RPTPσ has a novel heparin-binding site that transmits outside-in signals from extracellular HS.

***Aim 2*: To define the binding specificity of RPTPσ/ RPTPδ /RPTP-LAR to GAGs**

Hypothesis: RPTPσ / RPTP δ /RPTP-LAR recognize HS and CS GAGs in distinct ways.

***Aim 3*: To identify signaling molecules associating with RPTPσ upon GAG binding**

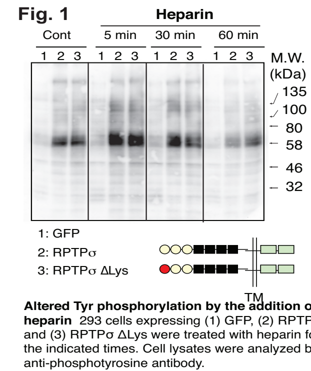
Hypothesis: Distinct sets of proteins are associating with RPTPσ in the cytoplasm upon HS and CS GAG binding, respectively.

Results

AIM 1

Heparin binding upregulated intracellular pTyr independently of known GAG binding site on RPTPσ

We investigated whether the complex formation between full length RPTPσ and GAGs impacts the receptor's enzymatic activity under physiological conditions (Fig. 1). When 293 cells expressing wild-type RPTPσ were treated with heparin, the intracellular pTyr level was transiently upregulated. Of particular interest is that the disruption of the Lys-loop within the first Ig domain (ΔLys mutant) did not abolish pTyr upregulation. Although this Lys-loop has been reported to be a sole GAG binding site in RPTPσ[3,4], this finding strongly suggests the presence of other heparin binding domain(s) in the extracellular portion of RPTPσ.



Altered Tyr phosphorylation by the addition of heparin 293 cells expressing (1) GFP, (2) RPTPσ, and (3) RPTPσ ΔLys were treated with heparin for the indicated times. Cell lysates were analyzed by anti-phosphotyrosine antibody.

Sulfation-specific binding of RPTPσ to GAGs

To biochemically analyze the interaction between RPTPσ and GAGs, ECD of RPTPσ were expressed as a fusion protein with alkaline phosphatase (AP) and solid phase binding assays were performed with immobilized heparin, CS, and DS GAGs. Scatchard plot analysis revealed a strong binding of RPTPσ to heparin with a K_d of 0.760 nM, consistent with a previously reported value[5]. Among CS/DS GAGs analyzed, only CS-E and DS showed binding to RPTPσ with the typical nanomolar range of K_d values for biologically relevant ligand-receptor interactions.

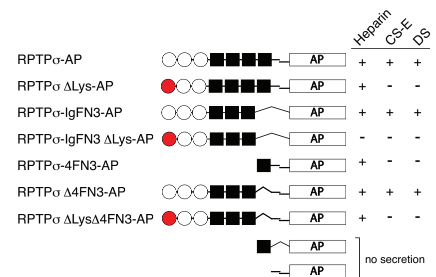


Fig. 2. Schematic structure of deletion mutants of RPTPσ and GAG binding

Identification of novel heparin-binding site on RPTPσ

While disruption of the Lys-loop abolished the binding of RPTPσ to CS-E and DS, heparin binding persisted (Fig. 2), consistent with Fig. 1. To further characterize the second binding domain, a series of deletion mutants were prepared and subjected to affinity chromatography to check their binding ability to heparin. Our finding strongly suggests that a novel binding site resides within the flanking regions of the fourth FNIII domain.

To identify this novel heparin binding site(s), "heparin protection" assays were performed[6]. Amino acid residues involved in the interaction with heparin were labeled by NHS-biotin after acetylation of non-essential Lys/Arg residues exposed to the surface of the molecule. Lys/Arg residues selected as potential heparin binding sites by proteomics analyses were then mutated to Ala individually and AP fusion proteins with these mutations were subjected to heparin affinity chromatography. We discovered 2 binding sites within the juxtamembrane domain: the positive charge stretches $R^{762}R^{763}R^{766}H^{767}R^{769}$ and $H^{809}R^{810}$. Disruption of either of these novel binding sites reduced the binding to heparin. Disruption of all 3 binding sites completely eliminated the binding.

Novel heparin-binding sites regulate the enzymatic activity of RPTPσ

Coles et al.[4,7] reported that HS binding to the common GAG-binding site in the 1st Ig domain on RPTPσ induced clustering of the extracellular region of RPTPσ, whereas CS binding did not. However, we found that both CS-E and heparin induced the clustering (data not shown). Thus, one question that arises here is whether these novel heparin-binding sites are involved in the altered pTyr upon heparin binding. While heparin induced a quick upregulation of pTyr through wild-type RPTPσ, but not the phosphatase deficient mutant, we observed very high level of baseline pTyr without heparin in cells expressing heparin-binding deficient mutant (Fig. 3). Addition of heparin did not alter the level of pTyr. This implies that elimination of all heparin-binding sites on RPTPσ caused the dysregulation of pTyr within the cells.

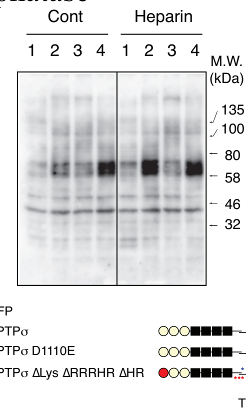


Fig.3. **Dysregulated pTyr by disruption of all heparin binding sites on RPTPσ** 293 cells expressing (1) GFP, (2) RPTPσ, (3) RPTPσ phosphatase-dead, and (4) RPTPσ triple mutant were treated with heparin and pTyr was examined by immunoblot.

AIM 2

Comparison of the binding specificity of RPTPσ/ RPTPδ /RPTP-LAR to GAGs

Because of the structural homology of the first two Ig domains among these receptors, it is tempting to speculate that both LAR and RPTPδ bind to HS and CS GAGs in the same way as RPTPσ, but thorough biochemical analyses are missing. We approached this issue with solid-phase binding assays as described above. Heparin, CS-E, and DS are recognized by all RPTPs with typical nanomolar range of K_ds (Table). Major CS GAGs in vivo (CS-A and CS-C) were not the ligands for RPTPs. Disruption of the Lys-loop responsible for GAG binding[4] within the 1st Ig domain gave us very unexpected results (Fig. 4): (1) RPTPδ binding to all GAGs was abolished by this mutation, (2) While RPTPσ and RPTP-LAR lost the activity to bind to CS-E and DS, they retained the binding to heparin. This indicates the presence of a novel binding site on RPTP-LAR as seen in RPTPσ. Further, the deletion of FNIII domains from RPTP-LAR reduced the binding to CS-E and DS, demonstrating the contribution of FNIII domains to GAG binding. Thus, there is a clear difference among RPTPs in their GAG binding.

	RPTPσ		RPTPδ		LAR	
	K_d (nM)	B_{max}	K_d (nM)	B_{max}	K_d (nM)	B_{max}
Heparin	0.7604 ± 0.1757	351.1± 22.19	0.603 ± 0.3106	138.8 ± 17.19	0.7953 ± 0.379	123.8 ± 3.829
CS-A	n.b.	n.b.	n.b.	n.b.	n.b.	n.b.
CS-C	n.b.	n.b.	n.b.	n.b.	n.b.	n.b.
CS-D	n.b.	n.b.	n.b.	n.b.	n.b.	n.b.
CS-E	3.052 ± 0.2929	360.4 ± 13.53	3.11 ± 0.6297	71.93 ± 10.2	4.032 ± 0.8662	225.8 ± 36.62
DS	4.022 ± 0.3153	340.6 ± 11.37	1.946 ± 0.3148	34.98 ± 3.022	6.173 ± 3.885	96.62 ± 3.829

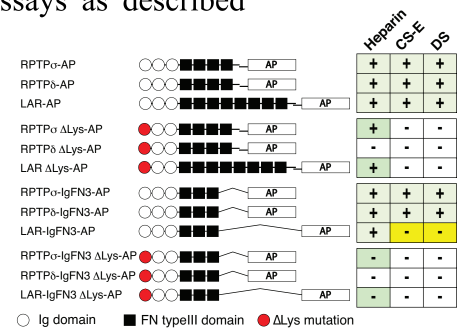


Fig. 4. Schematic structure of deletion mutants of RPTPs and GAG binding.

AIM 3

Identification of signaling molecules associating with RPTPσ upon GAG binding

To identify signaling molecules associating with RPTPσ, proximity labeling technique with APEX2 was utilized[8]. 293 cells expressing RPTPσ-APEX2 (full length of RPTPσ with APEX2 at its C-terminus) were treated with heparin for 10 min and biotinylation was induced by H_2O_2 for 1 min. Biotinylated proteins were isolated by Streptavidin beads. The peptides generated by

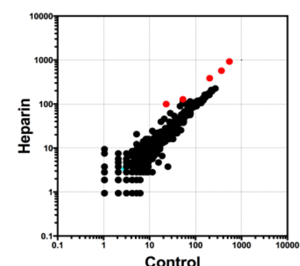


Fig. 5 Scores plot of all measured label-free quantitation values. Red circles indicate heparin specific.

tryptic digestion were identified by LCMS. We have identified 880 proteins (Fig. 5). MYH9, MYH10, ACTB, SPTN1, POTEE, and MPRIP were specific for heparin treatment. We are now in the process of confirming the heparin-induced associaiton of individual proteins with RPTPσ by the use of the NanoBit™ system, where live cell protein:protein interaction can be monitored through luminescence intensity under physiological conditions.

Discussion

The interactions of proteoglycan GAG chains with Type IIa tyrosine phosphatases are of considerable importance during brain development and in regenerative neurobiology. Structural analyses have indicated that the GAG binding site in the first Ig domain mediates these interactions[4]. Although there is a very high structural homology in the first two Ig domains among members of Type IIa tyrosine phosphatases (RPTPσ/RPTPδ/LAR), we have discovered the clear difference in the GAG binding (AIM 2). While RPTPδ harbors a sole binding site in the 1st Ig domain, contributions of other domains to GAG binding are seen in RPTPσ/LAR.

The assumption that the Lys-loop in the 1st Ig domain is a sole GAG binding site in RPTPσ has led to the conundrum that both HS and CS bind to this site but produce opposite actions. We now provide evidence for additional binding sites, specific to HS, in the juxtamembrane domain (AIM 1). Further, we show the involvement of the newly identified heparin-binding sites in the signal transduction. We speculate that the juxtamembrane domain, including the novel heparin binding sites, regulates the intracellular phosphorylation state coordinated by RPTPσ (Figure in the abstract). In this model, HSPGs on the same cell surface interact in *cis* with RPTPσ through the novel binding sites near the transmembrane domain and regulate the enzymatic activity of RPTPσ. Native HSPGs in the glycocalyx may limit the complex formation with RPTPσ[9], resulting in the regulated cytoplasmic phosphorylation state. This notion is supported by the fact that heparinase III treatment increased the sensitivity to exogenous heparin (data not shown). When HS GAGs with specific sulfation patterns interact with RPTPσ through this region, certain sets of protein phosphorylation are altered in the cells and the ligands work as permissive cues. When CS/DS GAGs are presented in *trans*, the binding is through the first Ig domain far from the transmembrane domain and triggers the changes in phosphorylation of distinct sets of proteins within the cells, resulting in the recognition of CS/DS as inhibitory cues by RPTPσ.

We focused on the alteration of intracellular pTyr induced by GAG binding. It is not clear whether these changes are derived from direct actions of RPTPσ phosphatase or indirectly, but phosphatase activity in the first cytoplasmic domain is required for intracellular signaling by heparin (Fig. 3). There are several reports showing altered phosphorylation of endogenous cytoplasmic proteins through RPTPσ[10,11]. However, it is still possible that the intracellular domain of RPTPσ functions as a scaffold protein. A deficient mutant of the Drosophila type IIa RPTP dLAR can be rescued by a catalytically inactive receptor but not by a dLAR mutant lacking the second inactive phosphatase domain[12]. To fully understand multiple ligand interactions and signaling pathways leading

to distinct functional outcomes, additional effort is needed to clarify endogenous substrates for RPTPσ as well as non-substrates associating with the cytoplasmic region of the molecule (AIM 3).

Although critical, HSPGs and CSPGs are not the sole physiological binding partners of type IIa RPTPs, and recent work has demonstrated novel roles of RPTPs as presynaptic proteins that interact with multiple postsynaptic partners to mediate synapse organization[13]. Our results suggest that synapse organization through presynaptic RPTPs with their postsynaptic partners could be modulated by HSPGs and CSPGs via several modules in their extracellular domains.

Many serious injuries to the mammalian CNS, such as spinal cord injury, traumatic brain injury or stroke, result in permanent disabilities. There are currently no drug therapies available that improve the very limited natural recovery from spinal cord injuries. While CSPG's inhibitory role in neuronal regeneration is without doubt, the blocking of extracellular inhibitory influences alone is not sufficient to allow the majority of injured axons to regenerate robustly, indicating that diminished intrinsic regenerative ability critically underlies regeneration failure[14]. A successful regenerative strategy[15] is likely to require combinatorial modulations that could remove extracellular inhibitory influences, promote the remyelination of regenerating axons, and stimulate the synaptic integration of regenerating axons into the pre-existing circuits for neuronal functions. Thus, the information obtained in this research project will contribute to design novel strategies to speed up axon regeneration.

List of publications:

Katagiri, Y., Morgan, A.A., Yu, P., Bangayan, N.J., Junka, R., and Geller, H.M. (2018) Identification of novel binding sites for heparin in receptor protein-tyrosine phosphatase (RPTPσ): Implications for proteoglycan signaling. *J. Biol. Chem.* 293:11639-11647.

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09/04/2018

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