A novel regulatory mechanism of the BMP signaling pathway

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Abstract

The BMP signaling pathway is a pivotal morphogenetic signal involved in a wide spectrum of cellular processes. The fact that the number of ligands far exceeds the number of receptors, and how a limited canonical pathway can accomplish pleiotropic effects demonstrate that the regulation of this pathway is, at present, poorly understood. In this study, we propose N-linked glycosylation as a specific regulatory mechanism of the BMP type 2 receptors (ACVR2A, ACVR2B and BMPR2). Computational screening for glycosylated asparagine residues in BMPR2 reveals three putative sites, which we show to be glycosylated by means of site-directed mutagenesis. Furthermore, we demonstrate that BMPR2 glycosylation is essential for ligand binding but that glycosylation of ACVR2A prevents binding. Collectively, our findings provide the first mechanistic insight into the regulation of the BMP signaling pathway through glycosylation of BMP type 2 receptors.

Summary

Numerous organismic processes such as embryonic development and bone growth are controlled by a cell regulatory mechanism known as the bone morphogenetic protein (BMP) pathway. This pathway is activated when a signaling protein binds to the membrane receptor, transmitting in turn an order to the nucleus. In an attempt to shed light on BMP pathway activation, we focused on receptor-bound sugar chains in view of their protein-specific signature. To study the role of individual sugar chains, we systematically blocked their function until we were able to pinpoint three key sites. We also assessed if these sugars affected the receptor's ligand-binding ability, and found that they promoted binding to the receptor in some cases and prevented binding in others. Thus, our findings provide the first mechanistic explanation for the BMP pathway regulation at a receptor-specific level.

1 Introduction

1.1 The TGF-β superfamily

The bone morphogenetic protein (BMP) signaling pathway plays a key role in the regulation of many cellular processes, such as embryo and cell growth, differentiation and apoptosis [1]. The BMP pathway has been observed to be necessary for the maintenance of vascular and bone homeostasis, leading to severe disease when mutations occur in members of the pathway [2].

The BMP ligands belong to the TGF-β superfamily, featuring many other proteins such as growth and differentiation factors (GDFs) that regulate cartilage and skeletal development [3], Activins (Acts) and inhibins (Inhs) that regulate pituitary hormone secretion [4], the Müllerian inhibiting substance (MIS) that determines sex during embryonic development [5] and bone morphogenetic proteins (BMPs) that regulate vertebrate development [6].

Receptor extracellular ligand binding is necessary for the activation of the BMP signaling pathway. Mechanistically, the initial activation of the pathway is achieved via the binding of transforming growth factor β-like (TGF-β-like) ligands to the Activin/TGF-β and BMP receptors, leading to cellular response through the regulation of target genes.

1.2 Structure and function of BMPs

BMPs have recently been under extensive study due to their implications in human disease. BMPs were originally described by Urist in 1965 [7], who observed that extracts of this protein had the ability to induce osteogenesis after intramuscular implantation. Since then, BMPs have been reported to contribute to a wide range of processes, such as cartilage development, osteogenesis and oocyte development [8].

BMPs bind to the extracellular domain (ECD) of the type II receptor (BMPR2, ACVR2A and ACVR2B) [9], which dimerizes with another receptor of its type. The dimeric type II

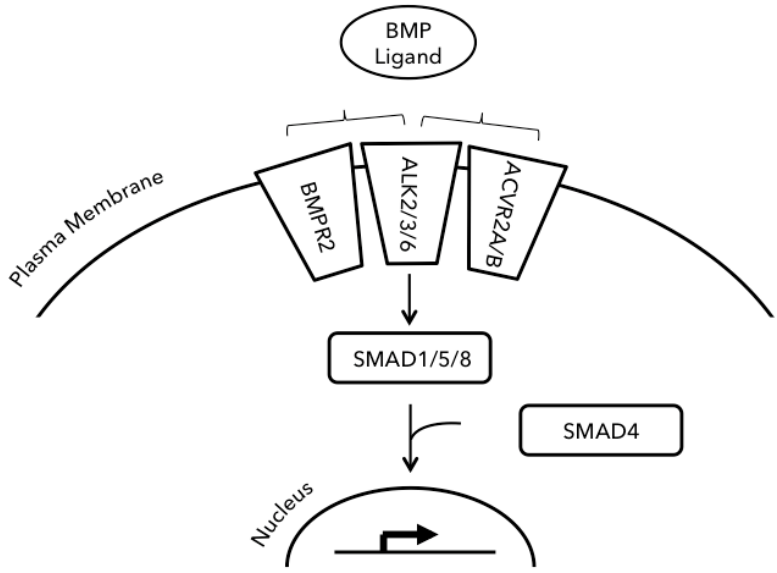

Figure 1: The activation of the BMP signaling pathway occurs when a BMP ligand triggers dimerization of type 1 (ALK2, 3, 6) and type 2 (BMPR2, ACVR2A, ACVR2B) receptors, leading to phosphorylation of SMAD1/5/8 and translocating to the nucleus together with SMAD4 for transcription of genes containing the SMAD binding elements.

receptors will phosphorylate the GS box of the type I receptors (ALK2, ALK3 and ALK6) [9], inducing the type I receptor to phosphorylate the C-terminus of several receptor-activated (RA) SMAD proteins (SMAD1, SMAD5 and SMAD8) [10]. The RA-SMAD complex will then bind to SMAD4 and translocate as a RA-SMAD/SMAD4 complex to the nucleus, facilitating the transcription of genes by binding to SMAD binding elements (SBEs) (Figure 1) [11].

The number of characterized BMP ligands outweighs the number of known receptors, giving rise to a competitive and synergistic receptor activation network. Competition for the bone morphogenetic protein 2 (BMP2) has been identified as a potent regulator of the canonical SMAD pathway (SMAD1/5/8) [12]. Several studies have reported that affinity of BMP2 to type 2 receptors ACVR2B and ACVR2A is higher than to BMPR2 [13, 14]. As ACVR2A and ACVR2B also bind to Activin, eliciting the receptor-activated SMAD2/3 canonical

pathway, which is often opposite in effect to SMAD1/5/8, it is possible that BMPR2 has a regulatory role in SMAD signaling based on BMP and Activin competition.

However, many of these studies have been performed using bacterially-expressed proteins, which might lead to artifactual differences based on altered posttranslational modifications. One such possible modification is N-linked glycosylation, which does not occur in bacteria.

1.3 The role of glycosylation in BMP binding kinetics

Glycosylation is a protein posttranslational modification based on the addition of polysaccharides to key residues, playing a role in protein structure and function [15]. The importance of glycosylation in determining a protein's properties has been shown to be critical in a number of cases, such as hormones and cytokines [15]. In the case of secreted signaling proteins, glycosylation can alter their conformation and binding kinetics, and thus control pathway activation [15].

N-linked glycolysation occurs when a monosaccharide, N-acetylglucosamine, attaches to a nitrogen molecule in an asparagine residue. In eukaryotes, glycosylation takes place in the endoplasmic reticulum (ER) lumen, for a further cleavage to the cellular membrane [16].

While N-linked glycosylation has been well characterized in a number of proteins, its effect on the type 2 BMP receptor is still to be clarified. Kang *et al* [17] showed that a non-N-glycosylated pro-VEGF region significantly reduced VEGF secretion in *Saccharomyces cerevisiae*, demonstrating that N-linked glycosylation has a bearing in protein stability. In another study, Zheng *et al* [18] found that thermal stability and susceptibility to degradation by papain were dependent on glycosylation status, although protein secondary and tertiary structure were unchanged.

1.4 Research approach

In this study, we aim to explore the structural and functional repercussions of aberrant or null BMP type 2 receptor glycosylation in BMP2 binding. Using several BMPR2 models containing single, double and triple substitutions in glycosylated asparagine residues, we assess BMP2 binding to BMPR2, as well as to the other type II receptors (ACVR2A and ACVR2B) to further understand the specific role of glycosylation at the individual receptor level. We hope our findings will shed light on BMP type 2 receptor function, enabling a clearer understanding of their complex and highly specific regulatory mechanism.

2 Methods

2.1 *hBMPR2* plasmid constructions

Prior to the start of RSI, several plasmids were generated in order to study the effects of site-directed mutagenesis on hBMPR2 function. A shuttle plasmid encoding the human BMPR2 was subcloned into the carbenicillin-resistant pcDNA3.1/V5-His-TOPO expression vector (Invitrogen), which appends C-terminal V5 and His epitope tags in-frame, via standard PCR using GoTag HotStart DNA Polymerase (Promega). The obtained plasmids were *hBMPR2*, *hBMPR2- N55Q*, *hBMPR2 -N110Q*, *hBMPR2- N126Q*, *hBMPR2- N55Q/ N110Q*, *hBMPR2- N55Q/ N110Q*, *hBMPR2- N55Q/ N126Q*, *hBMPR2- N110Q/ N126Q* and *hBMPR2- N55Q/ N110Q/ N126Q*. The primers used for plasmid sequencing plasmids are shown in the appendix (Appendix A.1).

2.2 W20 transfections

Mutant species of hBMPR2 were obtained via the transfection of W20 cells with plasmids containing the single, double and triple mutant *hBMPR2* sequences. Before transfecting

the cells, *Escherichia coli* bacteria were transformed in order to propagate the plasmids. To effectuate the transformation, we firstly transfered 50μl of bacteria and added 1μg of plasmid DNA. To permeabilize the bacterial membrane, the culture was heated up to 42^{o}C during 20 seconds and selection was performed in carbenicillin-containing media. The culture was further incubated at 32^{o}C with shaking, and 100μl of the bacteria were then transferred into a new agar plate for further growth overnight. After incubation, the cultures were pelleted at 13000rpm and the plasmids isolated following the QIAprep Spin MiniPrep kit (QIAGEN) instructions.

The transfection was performed using the X-tremeGENE 9 DNA Transfection Reagent (Roche) on W20 cells, previously passaged in 10% DMEM. To perform the transfection, the X-tremeGENE 9 DNA Transfection Reagent was vortexed and 3μl of the reagent was added to 100μl of serum-free medium (to a concentration of 3:1 reagent per DNA ratio). At this point, 1μg of DNA was added to the 100μl of diluted reagent, and the solution was incubated for 15 minutes at room temperature. As the W20 cells were grown in a 25cm^2 dish, 300μl of the diluted reagent was further added to the cell culture vessels in a dropwise manner without removal of the media.

Lysis of the cells was performed using RIPA buffer 72 hours after transfection, where the proteins were retrieved in the supernatant phase and subsequently prepared for western blotting.

2.3 Immunoblotting

Western blots were performed on lysates from transfected W20 cells and BMPR2-ECD/Fc chimeras. Proteins were resolved on Novex Tris Glycine gels (Invitrogen) and transferred to Amersham Hybond ECL nitrocellulose membranes (GE Healthcare). After blocking in 10% milk, the following primary antibodies were applied in 5% milk overnight at 4^{o}C or 1 hour at room temperature: mouse-produced anti-His tag (abcam, ab9108), mouse-produced anti-V5

tag (abcam, ab27671), mouse-produced anti-BMP2 (BD Systems, MAB3551) and rabbit-produced anti-HSP90 (Santa Cruz, sc-7947). After incubation with the primary antibody and washing the membrane with phosphate buffer saline Tween (PBST), the appropriate polyclonal secondary antibodies was utilized: either peroxidase-labelled anti-mouse (KPL, 04-18-06) or horseradish-linked anti-rabbit (Cell Signalling Technology, 7074). Immunoblots were placed in the developing buffer provided in the Femto kit by Thermo, for analysis using the Syngene PXi system.

2.4 PNGase-F treatment assays

The peptide-N-glycosidase- F (PNGase-F) (P0704S, BioLabs) is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, disassociating the glycan from the asparagine residue thus deglycosylating it. The PNGase-F was used in the treatment of the human BMPR2-ECD/Fc chimera (811-BR-100, RD Systems), the human ACVR2A-ECD/Fc chimera (340-R2-100, RD Systems), and the human ACVR2B-ECD/Fc chimera (339-RB-100, RD Systems), containing a 6-His tag, as well as the V5-tagged BMPR2 mutants.

2.5 BMP2 pulldown assays

The BMP2 pulldown assays were performed using Dynabeads Protein G (novex by Life Technologies). An optimization assay was performed to determine the levels of background BMP2 in the pulldown assay, which determined that overnight incubation at 4^oC then transfering to a new tube was the optimum treatment (see Appendix A.2). The samples prepared for the pulldown experiments went in duplicates, in which 500ng of the assessed receptor ECD were added to a solution containing 2μl of Buffer G7, 2μl of 10% NP40, adjusted with water up to 20μl with either 1μl of PNGase-F or none, then incubated for 1 hour at 37^oC. Dynabeads

were vortexed, 10μl transferred into clean tubes and washed twice on the magnet with WB buffer (PBST at 0.02% Tween 20). Subsequently, water was added to the receptor-ECD sample adjusting the volume up to 200μl for transfer to the tubes containing the beads, to then be incubated at room temperature with rotation for 30 minutes. After incubation, the samples were washed twice on the magnet with PBS and 200μl of PBS were added into each replicate and 100μg of BMP2 exclusively into the second. The new mixture was transferred into a new tube and incubated overnight at 4^{o}C with rocking. After incubation, the supernatant was removed while placing the tubes on the magnet and the beads were washed five times with 200μl of PBS. Finally, the beads were resuspended in 100μl of PBS and transfered into a new tube, where the supernatant was removed on the magnet. At this point, 20μl of denaturing sample buffer was added and the samples proceeded to western blotting.

2.6 Putative glycosylated sites identification and structural analysis

The asparagine residues were identified on the type II receptors (ACVR2A, ACVR2B and BMPR2) using the online server NetNGlyc 1.0 [19] guided by the Asn-Xaa-Ser/Thr sequon, including Asn-Pro-Ser/Thr. The receptor's FASTA sequences were obtained from the Protein Data Bank (PDB), using a model for BMPR2 (PDB ID: 2HLQ [20]), ACVR2A (PDB ID: 2H62 [21]) and for ACVR2B (PDB ID: 1BTE [22]). The residue alignment was performed using Standard Protein BLAST [23], and the figures were generated using PyMOL [24].

3 Results

3.1 Structural analysis of type 2 receptors reveals the location of glycosylated sites

Structural profiling and search for putative glycosylation sites in the type II receptors ACVR2A, ACVR2B and BMPR2 showed that glycosylated asparagines are distributed in a receptor-specific manner (Figure 2). In the case of the Activin receptors, two homologous pairs of glycosylated asparagines in the ECD were discovered: N43-N66 in the case of ACVR2A and N42-N65 in ACVR2B. Interestingly, an asparagine residue situated in the ligand-receptor interface (N110) was found only in BMPR2, suggesting a glycosylation-induced specificity mechanism for ligand binding.

3.2 BMPR2 is glycosylated

To assess native BMPR2 glycosylation, we originally used an endogenous BMPR2 from W20 cells, which we treated with PNGase-F to observe if there were changes in molecular size owing to the existence of glycosylated sites. When treated with PNGase-F and immunoblotted, a clear reduction in size was observed (Figure 3): when endogenous BMPR2 was run without PNGase-F treatment, the approximate molecular weight was 150kDa, while the replicate treated with PNGase-F showed a band in 135kDa. As PNGase-F only cleaves between the innermost GlcNAc and the extracellular asparagine residue of high mannose, there are no other structural traits that may have been affected with the exception of N-linked glycans, demonstrating that BMPR2 is a glycoprotein.

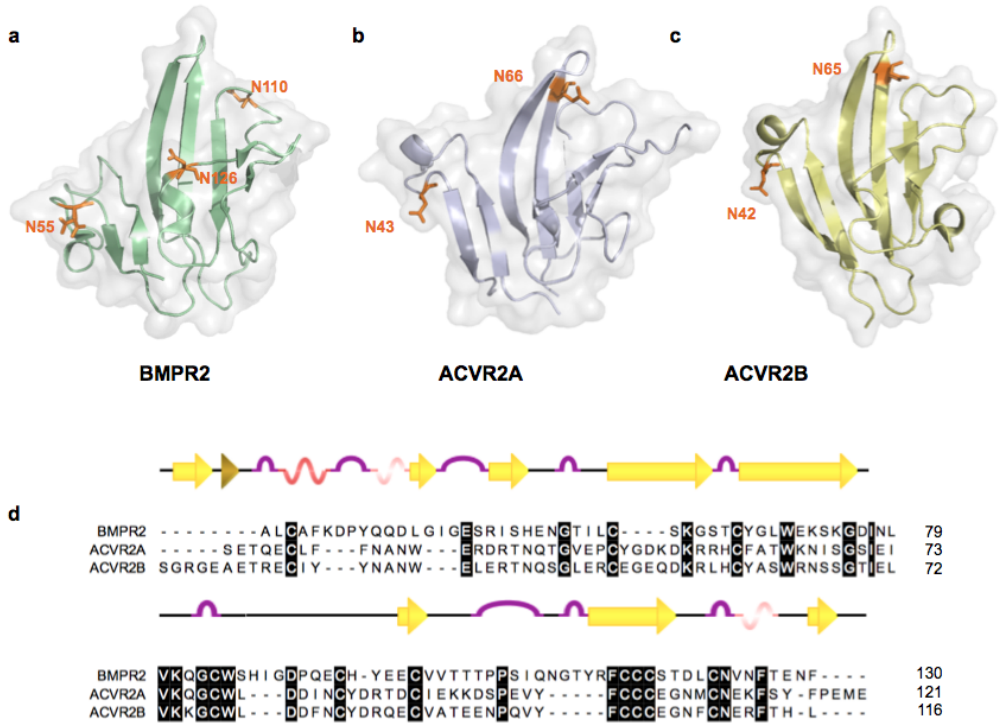

Figure 2: Representation of the three type II receptors (ACVR2A, ACVR2B and BMPR2). Location of glycosylated asparagine residues in (a), BMPR2, (b), ACVR2A and (c), ACVR2B. In (d), the residue sequences of the type II receptor's ECD are compared.

3.3 BMPR2 glycosylation occurs in three key asparagine residues in the BMPR2 extracellular domain

In order to explore the importance of the three putative N-glycosylated sites in the BMPR2's ECD, we used several recombinant versions of BMPR2 where these asparagine residues were substituted with a non-glycan binding residue such as glutamine by means of site-directed mutagenesis. The models used either contained single asparagine mutations (N55Q, N110Q and N126Q), double mutantions (N55Q/N110Q, N55Q/N126Q and N110Q/N126Q) or a triple mutation (N55Q/N110Q/N126Q).

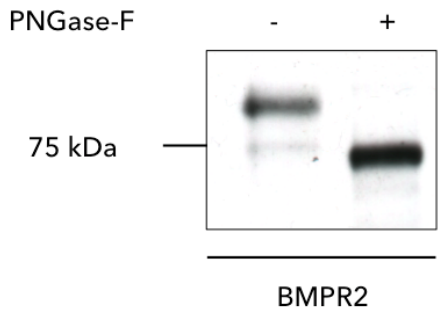

Figure 3: Treatment of BMPR2 with PNGase-F results in a molecular weight reduction. The western blot shows immunoblotting of the native, endogenous BMPR2. In order to differentiate between the cell's WT BMPR2 and our mutant versions of BMPR2, subsequent experiments incorporated a V5 epitope tag which was targeted during immunoblotting.

In the case of the individual single mutants, we observed that the BMPR2-N55Q and BMPR2-N126Q showed a lowered expression, if any, while the N110Q was correctly expressed and showed a band running lower than the WT BMPR2 (Figure 4).

In order to test if these three asparagine residues were the contributors of the BMPR2 glycosylation, the mutants were treated with PNGase-F. Our results show that the triple mutant band does not manifest any displacement after PNGase-F treatment, showing that these three sites monopolize BMPR2 N-linked glycosylation (Figure 5). The BMPR2 model bearing an active N126 but N55Q/N110Q substitutions showed a notable weight decrease when treated with PNGase-F (Figure 3), suggesting that N126 may be a key site for BMPR2 glycosylation.

Surprisingly, our data shows that when N110 is glycosylated but either one or both N55 and N126 are non-glycosylated, there is no BMPR2 expression (Figures 4 and 5). We hypothesize that this may be due to a specific BMPR2 degradation response mediated by N110 glycosylation, suggesting that this residue may be key in BMPR2 targeting for degradation in the asbsence of glycosylation at N55 and N126.

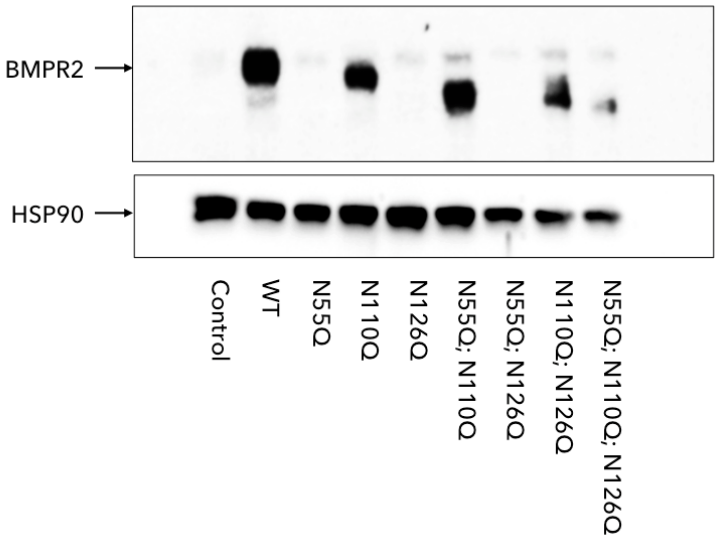

Figure 4: Mutated recombinant BMPR2 species bearing single, double and triple mutations in glycosylated sites reveal distinct effects in the protein. As a control, a lysate from W20 cells transfected with an empty plasmid was run in the western blot, demonstrating specificity of V5 antibody. Additionally, HSP90 was used as a loading control to ensure that protein levels were not general in the sample.

3.4 BMPR2 glycosylation is essential for BMP2 binding

To explore the effect of BMPR2 N-linked glycosylation in ligand binding, we performed a pulldown assay for BMP2 using glycosylated and non-glycosylated recombinant BMPR2-ECDs. Our results show that the PNGase-F-treated BMPR2 is unable to bind BMP2 during the pulldown: while the correctly glycosylated version was able to efficiently bind BMP2 (Figure 6), the non-glycosylated species showed a 11.9% binding efficiency relative to the glycosylated BMPR2. These results show that glycosylation of the BMPR2 plays a crucial role in BMP2 binding, adding another level of BMPR2 specificity during ligand binding and consequent activation.

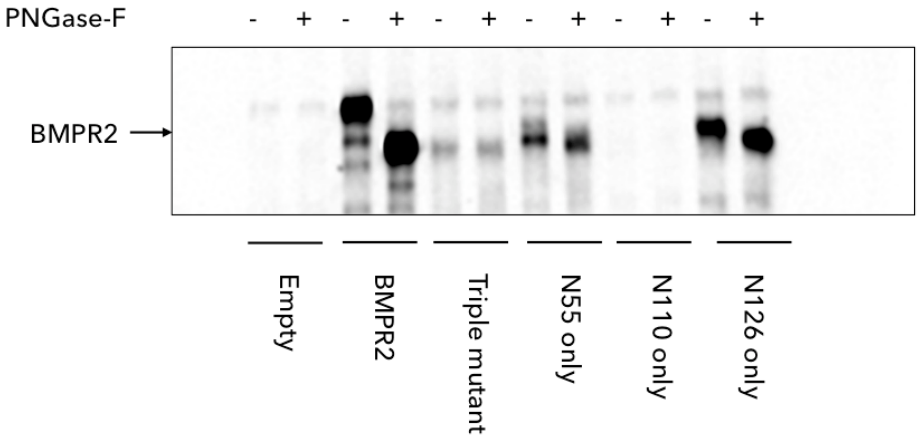

Figure 5: Combined glycosylation of two and three asparagines results in distinct BMPR2 outcomes. PNGase-F treatment of the negative control (empty), the recombinant WT BMPR2, the triple N55Q/N110Q/N126Q and the three double mutants having only one glycosylated site (N55, N110 and N126), all containing a V5-tag which was immunoblotted in the shown western blot.

3.5 Glycosylation acts as a BMP2-binding variable switch within the type II BMP2 receptors

To further understand if glycosylation is also necessary for BMP2 binding in the case of the other type II receptors (ACVR2A and ACVR2B), we performed a pulldown assay using the glycosylated and non-glycosylated species of ACVR2A and ACVR2B in the presence of BMP2. Strikingly, in the case of ACVR2A, the BMP2 pulldown efficiency was higher in the non-glycosylated form than when fully glycosylated, while no significant variations were observed in ACVR2B (Figure **??**). These results show that glycosylation has an enhancing effect in BMPR2 but an antagonist effect in ACVR2B, suggesting a potential and novel 'switch' regulating BMP2-specific pathway activation.

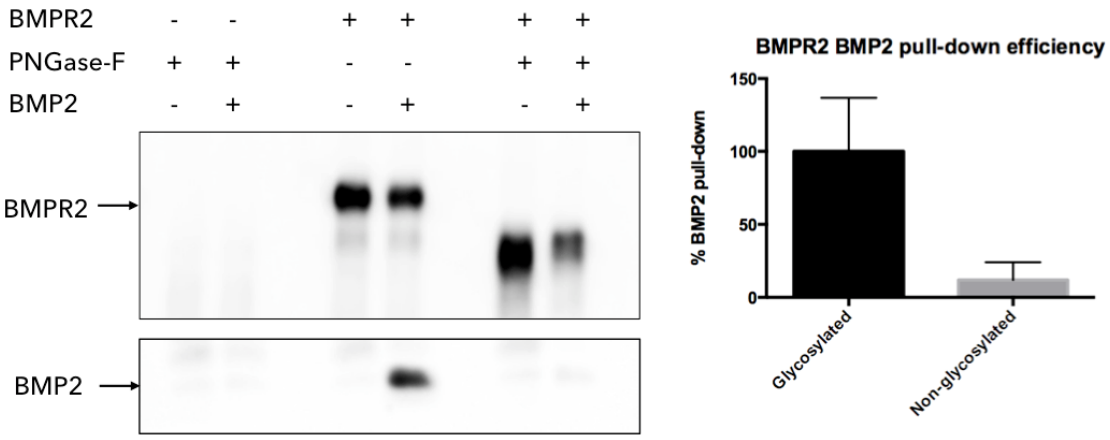

Figure 6: BMP2 is pulled down exclusively when BMPR2 is glycosylated. The displayed western blot shows four lanes, two of which have a fully glycosylated BMPR2 and the other show a non-glycosylated, PNGase-F-treated BMPR2. For each type, the pulldown was repeated with and without the presence of BMP2. These results show the levels of BMP2 attached during the pulldown and present in the western blot, which were compared to the average BMP2 pulldown in the glycosylated BMPR2 (P value=0.0443; df=2.326).

4 Conclusions

Altogether, this study provides a novel picture of the BMP regulatory pathway at the extracellular level based on N-linked glycosylation.

In order to determine if BMPR2 is a glycoprotein, we used PNGase-F to examine variations in molecular mass after treatment. We found out that there was a clear, 15kDa decrease in mass, demonstrating that BMPR2 is a glycoprotein. To identify the BMP type 2 receptor's ECD glycosylated sites, we used a computer-assisted search for Asn-Xaa-Ser/Thr sequons, resulting in the detection of three potential glycosylation sites in the BMPR2 (N55, N110 and N126). Then, to analyse the contribution of these asparagines, site-directed mutagenesis was used in order to generate individual, double and triple mutants on the putative glycosylation sites. The designed mutations were asparagine to glutamine substitutions, due to latter's inability to cleave glycosyl groups whilst preserving an almost identical structure to asparagine. By treatment with PNGase-F, we show that the three residues have distinct

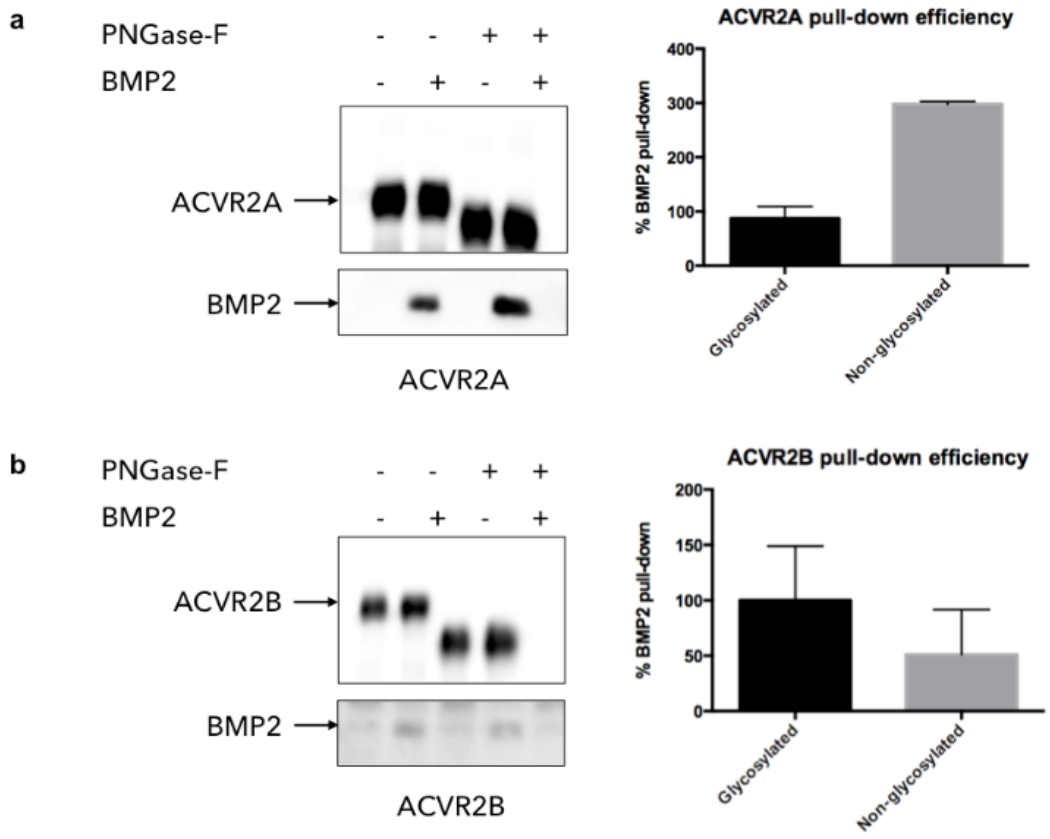

Figure 7: Glycosylation in ACVR2 receptors, viz. (a) the ACVR2A and (b) the ACVR2B, has different effects in their ability to bind BMP2 (for (a), P value=0.0379; df=1.091. For (b), P value=0.3930; df=1.933). As the receptors assessed were the ECD/Fc chimeras containing a His-tag, primary immunoblotting was targeted to the His-tag.

contributions to BMPR2. As expected, the triple mutant showed no weight variation after PNGase-F treatment, demonstrating that these three sites are the only contributors to N-linked glycosylation. Interestingly, the mutant's expression profile in a W20 cell culture was unique for every BMPR2 model. When N110 remained glycosylated an either one or both of the other asparagine residues (N55 and N126) were mutated no BMPR2 was expressed, suggesting the role of N110 as a protein degradation regulator.

When studying the effect of BMPR2 glycosylation for BMP2 binding, we observed that the non-glycosylated species was unable to bind BMP2, demonstrating that BMPR2 glyco-

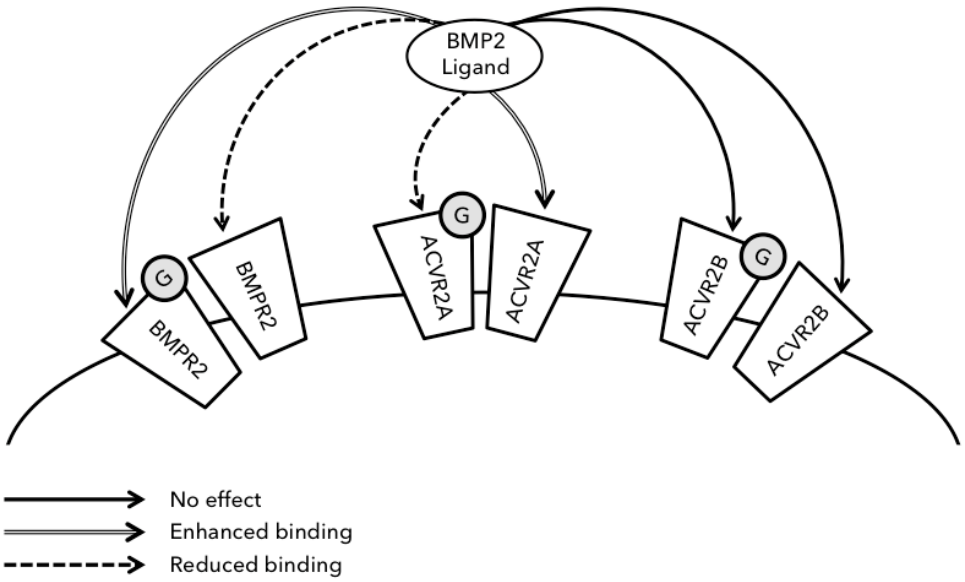

Figure 8: N-linked glycosylation of BMP type 2 receptors regulates the activation of the SMAD1/5/8 by modulating its affinity to BMP ligands. This hypothesis provides an insight into the specific ligand-mediated activation of the pathway at the BMP type 2 receptor level.

sylation is essential for BMP2 binding. However, when we when repeated the experiment on the other type 2 receptors (ACVR2A and ACVR2B), we observed a very distinct effect: while there was no apparent repercussion in ACVR2B, glycosylation was antagonistic in ACVR2A's efficiency to bind BMP2. This distinct effect of N-linked glycosylation between the different type 2 BMP receptors provides a novel mechanistic explanation for type-2-receptor ligand specificy.

5 Discussion

In this paper, we present a potential biological switch of the SMAD1/5/8 canonical pathway regulated by N-linked glycosylation at the type 2 receptor level. The complex activation of the BMP pathway has been since its discovery a mechanism yet to be fully clarified, in view of its implications in bone and cartilage growth [25, 26]. In this study, we directly assessed

the effect of BMP type 2 N-linked glycosylation on BMP2 binding, in order to determine if glycosylation plays a relevant role in BMP type 2 receptor activation by regulating ligand binding affinity. Our results show that N-linked glycosylation is required for BMPR2 to bind BMP2 but, strikingly, that it had an entirely different effect on the other type 2 receptors: while glycosylation did not affect ACVR2B BMP2 binding, it had an antagonistic effect on ACVR2A. Collectively, these results reveal the role of glycosylation as a potent regulator of the BMP pathway. Moreover, our characterization of the individual glycosylated asparagines provides a novel interpretation of pathologies driven by mutations in glycosylated BMPR2 asparagines. Heritable pulmonary arterial hypertension (HPAH) is the best example bearing these mutations [27], where N126 substitutions have been identified as highly detrimental in disease prognosis. Based on the implication of BMP type 2 receptors in numerous diseases such as renal fibrotic disease [28] and multiple myeloma [29], future studies should be directed towards the characterization of glycosylated and non-glycosylated type 2 receptor pools in different tissues, as well as the exploration of the implications in type 1 receptors, dimer formation and binding kinetics.

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A Appendix

A.1 Plasmid transfection

To obtain mutant versions of BMPR2 in order to explore the effect of individual asparagine substitutions, a series of overexpression systems using W20 cells were constructed prior to the start of RSI. The shuttle plasmid encoding human BMPR2 cDNA was obtained from Dr. James West at Vanderbilt University, and all plasmids were validated at the Dana Farber/Harvard Cancer Center Resource Core. During PCR, a series of plasmids were used in order to amplify the modificated BMPR2 (Table 1).

BMPR2 mutant	Primer	Primer sequence
hBMR2	Forward	5'-CGTGGCCCAGGGATGACTTAC-3'
	Reverse	5'-GAGACAATTCATTCCTATATCTTTAGACAC-3'
N55Q	Forward	5'-GAGAGTAGAATCTCTCATGAACAAGGGACAATCTTATGCTCAAAG-3'
	Reverse	5'-CTTTCGAGCATAATATTGTCCCTTGTTCATGAGAGATTCTACTCTC-3'
N110Q	Forward	5'-CCACTCCTCCCTCAATTCAGCAAGGAACATACCGTTTCTGC-3'
	Reverse	5'-GCAGAACAGGTATGTTCCTTGCTGAATTGAGGGAGGAGTGG-3'
N126Q	Forward	5'-GTAGCACAGATTTATGTAATGTCCAATTTACTGAGAATTTTCCACCTCC-3
	Reverse	5'GGAGGTGGAAAATTCTCAGTAAATTGGACATTACATAAATCTGTGCTAC-3'
N55Q/N110Q	Forward	5'-GAGAGTAGAATCTCTCATGAACAAGGGACAATCTTATGCTCAAAG-3'
	Reverse	5'-CTTTCGAGCATAATATTGTCCCTTGTTCATGAGAGATTCTACTCTC-3'
N55Q/N126Q	Forward	5'-GAGAGTAGAATCTCTCATGAACAAGGGACAATCTTATGCTCAAAG-3'
	Reverse	5'-CTTTCGAGCATAATATTGTCCCTTGTTCATGAGAGATTCTACTCTC-3'
N110Q/N126Q	Forward	5'-CCACTCCTCCCTCAATTCAGCAAGGAACATACCGTTTCTGC-3'
	Reverse	5'-GCAGAACAGGTATGTTCCTTGCTGAATTGAGGGAGGAGTGG-3'
Triple mutant	Forward	5'-GAGAGTAGAATCTCTCATGAACAAGGGACAATCTTATGCTCAAAG-3'
	Reverse	5'-CTTTCGAGCATAATATTGTCCCTTGTTCATGAGAGATTCTACTCTC-3'

Table 1: Plasmids used during the transfections in the BMPR2 overexpression system

A.2 BMP2 pull-down optimization

In order to ensure that the pull down would not show BMP2 background, we performed an optimization assay using the Dynabeads and BMP2 in several different incubation conditions. We found that the lowest background BMP2 apperared in the 4^oC incubation then trasnfered to a new tube (1.16%), and therefore this was the incubation condition used in the pull-down assays.

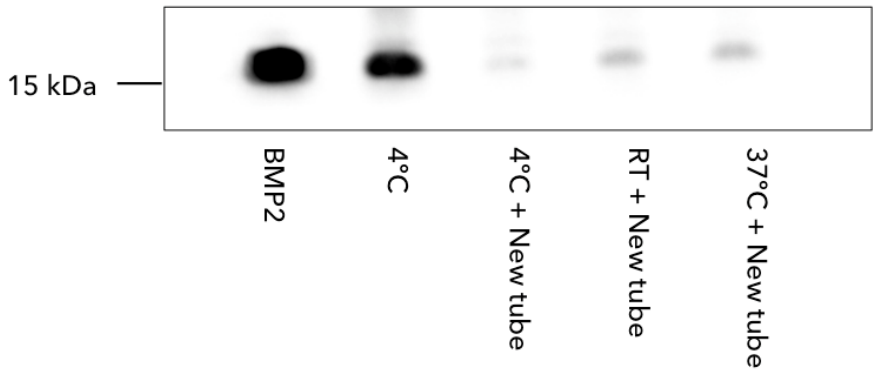

Figure 9: BMP2 background in the BMPR2-specific pull-down using three different conditions

Condition	4^oC old tube	4^oC new tube	RT new tube	4^oC new tube
Pull-down	59.75%	1.16%	5.71%	3.87%

Table 2: Percentage pull-down relative to BMP2 control, added at an equal amount