G6b-B: a new receptor for platelet production and function

Untangling post-translational modifications using mass spectrometry enables structure determination in complex with ligand

PEAK PROTEINS

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Introduction

G6b-B is an inhibitory receptor that is highly expressed on mature megakaryocytes and platelets (Senis et al, 2007). It is a single pass, type 1 membrane protein containing an immunoreceptor tyrosine-based inhibition motif. KO mouse models and human mutations show severe macrothrombocytopenia, demonstrating the importance in platelet biology (Mazharian et al, 2012, Hofman et al, 2018). The extracellular domain (ECD) is a single Ig domain that is glycosylated and very positively charged. The physiological ligand remains unknown although it is known to bind to sulphated oligosaccharides such as heparan sulphate and heparin. In this study we undertook to solve the crystal structure of the ECD of G6b-B in complex with a sulphated oligosaccharide ligand. A key aspect of the ultimate success of the study was the ability to analyse and engineer post-translational modifications, especially glycosylation.

Expression and Purification

G6b-B ECD was expressed transiently in HEK293-6E cells (Durocher et al, 2002) and purified from the culture medium by cation exchange and size exclusion chromatography. LC-MS analysis of this material revealed multiple peaks at ~15kDa indicative of N-glycosylation and a species that was 948 Da higher than the predicted mass. When the sole N-linked site was mutated a single species was observed on SDS-PAGE and LCMS, again +948 Da. This mass is consistent with a common O-linked tetrasaccharide. [N32D]G6b-B did not crystallise by itself but, when complexed with Fab, crystallised over a period of approximately two months to yield crystals that diffracted only weakly.

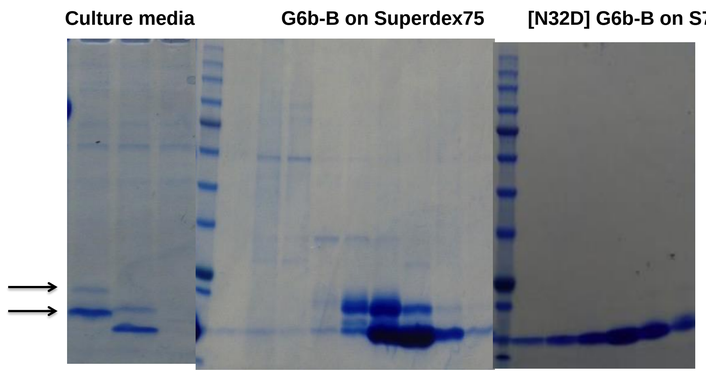


Figure 1: Expression and purification of G6b-B ECD. Engineering of N-linked glycosylation site results in a single species of +948 Da.

Analysis of O-linked glycosylation

The bioinformatics package NETOGlyc 4.0 suggested four potential O-linked sites in a chymotryptic peptide whose sequence is ASSSGTPTVPPLQPF. TOF-MS of a chymotryptic digest revealed a peak eluting at 11.5 minutes that confirmed this peptide as being O-glycosylated with a 3+ ion of 811 Da. In the same peak the un-glycosylated 2+ ion (743) along with a free sugar at 948 Da were also observed, probably due to in-source fragmentation.

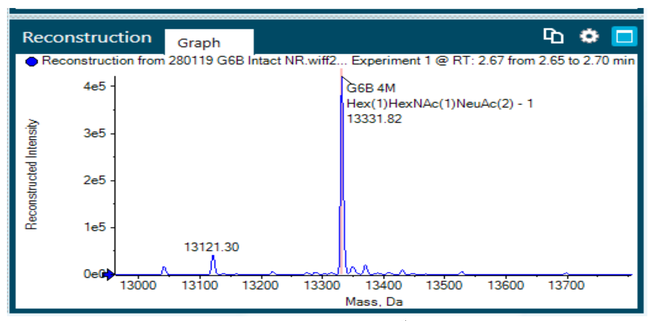


Figure 2a: Intact mass without reduction. Observed mass suggests two disulphides and O-glycosylation

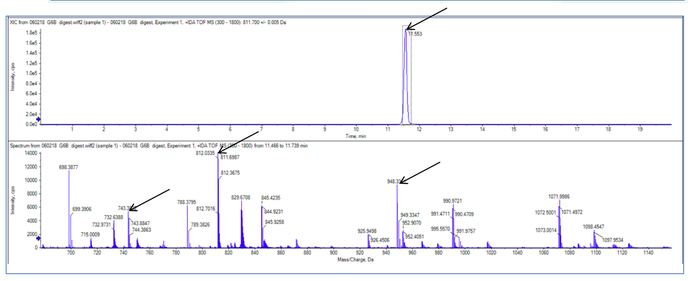


Figure 2b: TOF-MS analysis of chymotryptic digest

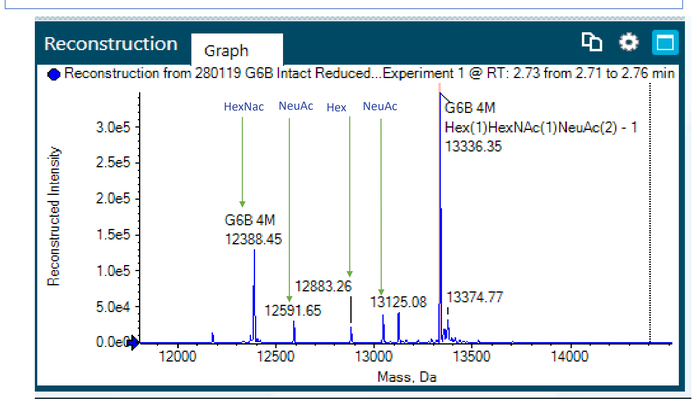
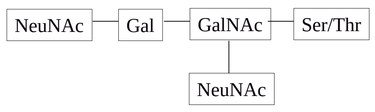


Fig 3: Intact mass with reduction. Breakdown of tetrasaccharide confirmed structure shown below

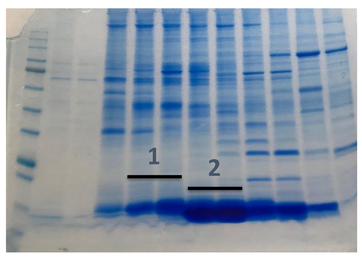


Mutation of potential O-linked residues

Table 1: G6b-B mutants and mass analysis

Mutation (All have N32D)	Predicted mass +O-glycosylation	Observed mass
S67A	13398	13394
S68A	13398	13394
S69A	13398	13394
T71A	13384	13380
T73A	13384	1=13380 2=12432
4M(AAAAT)	13336	13332
5M(AAAAA)	13306	12354

Fig 4: Cation exchange purification of [T73A]G6b-B



The mutational analysis reveals that the primary site of O-glycosylation is T73. However it is clear that even when this site is mutated to Ala some O-glycosylation can occur. This heterogeneity of position might explain difficulties in crystallisation.

Crystallisation and Structure Solution

Fig 5: Crystals obtained using '4M'-G6b-B

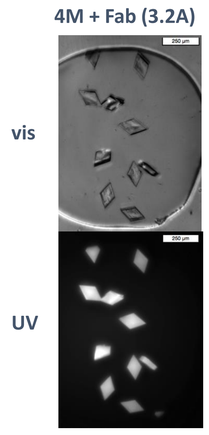
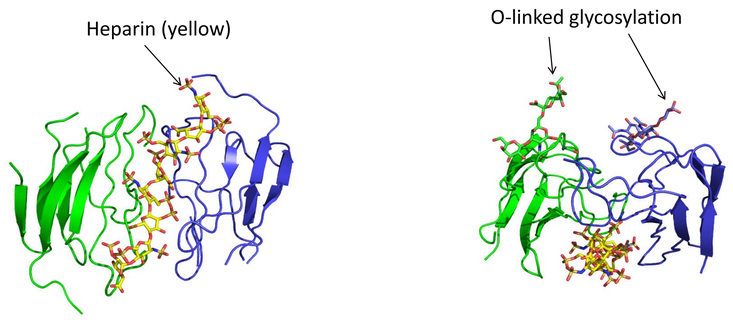


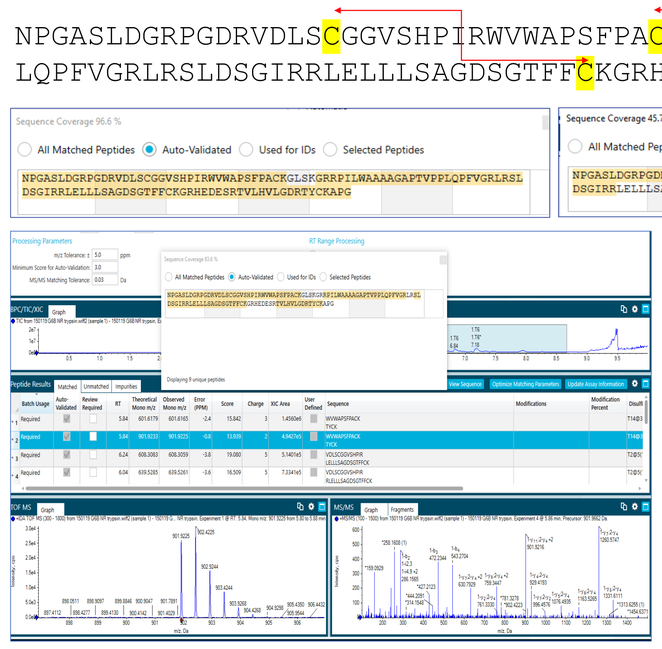
Fig 6: Structure of G6b-B with heparin based dodeca-saccharide reveals dimer formation around ligand.



Mapping of disulphide bonds using mass spectrometry

The disulphide bonding pattern of G6b-B could not be unambiguously defined from the structure. We therefore set out to identify the pattern using mass spectrometry. The suggested pattern is shown in a), the tryptic peptide maps under reducing and non-reducing conditions in b) and the full MS/MS analysis in c) together with a table of the linked peptides.

Fig 7: MS/MS analysis of disulphide bonds. All MS analysis was performed on the Sciex X500B system coupled to a Sciex Exion LC. Data was interpreted using Sciex BioToolKit or BioPharmaView software.



Sequence	Charge
WVWAPSFPACK TYCK	3
WVWAPSFPACK TYCK	2
VDLSCGGVSHPIR LELLLSAGDSGTFFCK	5
VDLSCGGVSHPIR RLELLLSAGDSGTFFCK	5

Conclusions

- **Mass spectrometric identification of the identity and location of glycosylation sites was critical to engineering a protein suitable for crystallisation**
- **The structure reveals a ligand induced dimer**
- **MS/MS analysis of a tryptic digest also unambiguously identified the disulphide bonds**

References

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