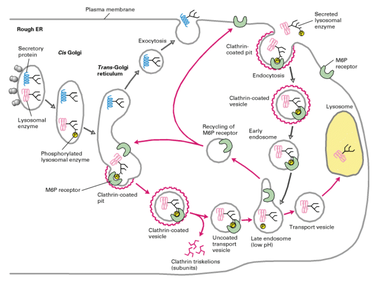
P-257

Hyperactive GlcNAc-1-Phosphotransferase (S1S3 PTase) Dramatically Alters Glycosylation of Lysosomal Enzymes Leading to Enhanced Phosphorylation for Improved CI-MPR Binding

Kylie Gray[1], Riley Marcinczyk[1], Jonathan Roberts[1], Uday Wanninayake[1], Vaughn Weaver[1], Michael DiGruccio[1], Madison Chao[2], Nastry Brignol[2], Osman Sheikh[2], Russell Gotschall[1]

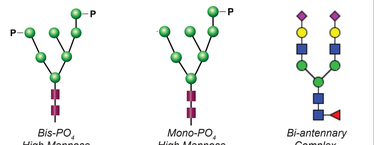
[1]M6P Therapeutics, St. Louis, MO 63108; [2]Amicus Therapeutics, Philadelphia, PA 19104

CI-MPR is the Main Receptor Responsible for Targeting Lysosomal Proteins to Lysosomes



Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 17.7

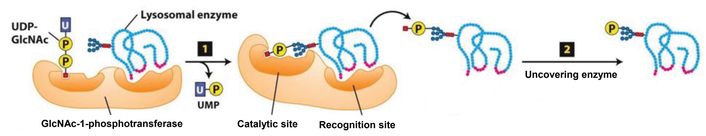
Bis-phosphorylated glycans have ~3,000x greater affinity for the CI-MPR than Mono-phosphorylated glycans



Ligand	Binding affinity (Apparent K_D; nM)*
Bis-PO_4 High Mannose N-glycan	2
Mono-PO_4 High Mannose N-glycan	>6000
Complex N-glycan	No binding

**Adapted from Tong el al. 1989*

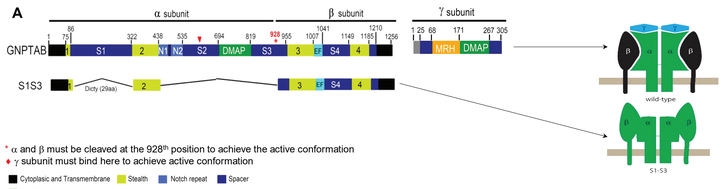
Soluble Lysosomal Enzymes Are Post-Translationally Modified to Contain M6P

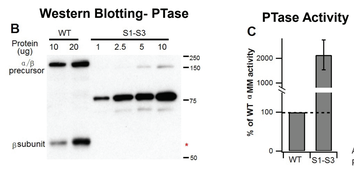


Kang et al. Mutations in the Lysosomal Enzyme – Targeting Pathway and Persistent Stuttering. N Engl J Med 2010;362:677-85.

- Step 1: GlcNAc-1-Phosphotransferase (PTase) catalyzes the transfer of GlcNAc-1-phosphate from UDP-GlcNAc onto certain terminal mannose residues of the N-linked oligosaccharides on enzymes destined for the lysosome
- Step 2: NAGPA (N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase), also known as Uncovering enzyme, removes covering GlcNAc group, thereby exposing mannose-6-phosphate (M6P)

S1S3 Truncated PTase has ~20X Specific Activity to Enable Better Phosphorylation of Lysosomal Enzymes





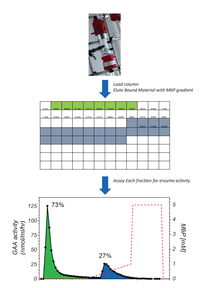
Molecular Therapy Methods & Clinical Development
Original Article
Engineering of GlcNAc-1-Phosphotransferase for Production of Highly Phosphorylated Lysosomal Enzymes for Enzyme Replacement Therapy
Lin Liu,[1] Wang-Sik Lee,[1] Balraj Doray,[1] and Stuart Kornfeld[1]
[1]Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA

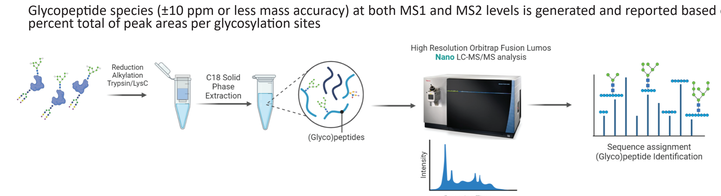
Adapted from Lin Liu et al. Engineering of GlcNAc-1-Phosphotransferase for Production of Highly Phosphorylated Lysosomal Enzymes for Enzyme **Replacement Therapy, Mol Ther Methods Clin Dev. 2017 Jun 16; 5: 59–65.**

Methodology

- *Protein Production and Purification*
  - Expressed in CHO (M011, M021 & M051) or Epi293 (M081) cells
  - Purified by affinity, anion exchange, and/or size exclusion chromatography
- *CI-MPR affinity chromatography*
  - Protein injected onto the CI-MPR affinity column and subsequently eluted with a M6P gradient
  - Fractions collected in 96 well plate
  - Protein amount in each fraction determined by activity
  - The relative amount of bound and unbound protein can be compared
- *CI-MPR binding kinetics*
  - Protein is added at various concentrations to a 96-well plate containing immobilized CI-MPR
  - Unbound protein is removed by plate washing
  - Activity of bound protein is determined and plotted against the binding concentration of the protein
  - Curve is fitted to one site specific binding equation in saturation binding equations in non-linear regression
  - Vmax and Kd are obtained
- *Glycan mapping*
  - All glycans are enzymatically released from reduced and alkylated recombinant protein
  - Released glycans are labeled with 2-AA or 2-AB fluorescent dye
  - Labeled glycans are quantified by rpHILIC-HPLC with fluorescent detection
- *Site-Specific Glycopeptide mapping*
  - Recombinant protein is denatured, reduced with TCEP, and alkylated with chloroacetamide
  - Peptides are generated by protease digestion with trypsin and Lys-C, cleaned-up by mixed-mode weak cation exchange solid phase extraction, and dried under vacuum
  - Peptides are separated by reverse phase NanoLC
  - Glycopeptide characterization is performed by Electron Transfer/High Energy Collision Dissociation fragmentation on an orbitrap Fusion Lumos Tribrid Mass Spectrometer
  - Raw data is uploaded into RefinerMS (Genedata Expressionist 16.5) and processed using an in-house human N-glycans library
  - Glycopeptide species (±10 ppm or less mass accuracy) at both MS1 and MS2 levels is generated and reported based on percent total of peak areas per glycosylation sites

CI-MPR Affinity Chromatography

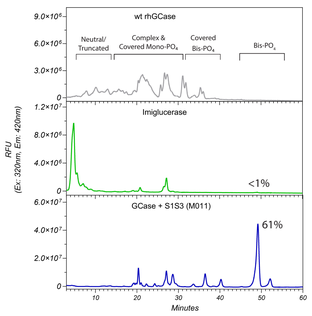




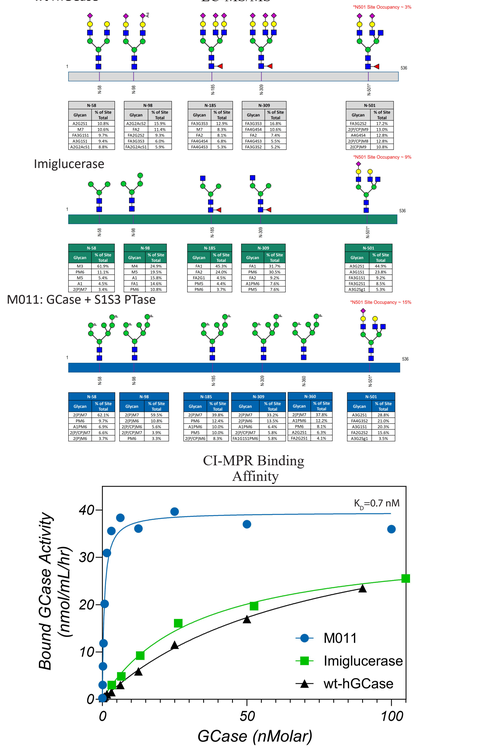
Glucocerebrosidase (M011) CI-MPR interaction and affinity is dramatically improved when co-expressed with S1S3 PTase

- β-Glucocerebrosidase (GCase) is a lysosomal enzyme that cleaves by hydrolysis the β-glycosidic linkage of the chemical glucocerebroside
- It is an ~60 kDa glycoprotein with 5-6 N-linked glycosylation sites (6th site engineered into M011 for added stability)
- Gaucher Disease is caused by reduced or absent GCase activity resulting in glucosylceramide accumulation in the liver, spleen, and macrophage lineage cells.

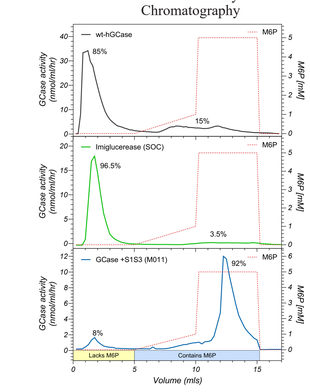
Fluorescently Labeled Glycan Chromatography



Site-Specific Glycopeptide Mapping LC-MS/MS



CI-MPR Affinity Chromatography

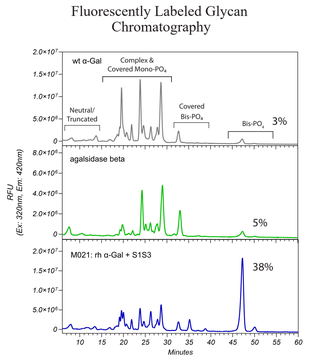


CI-MPR Binding Affinity

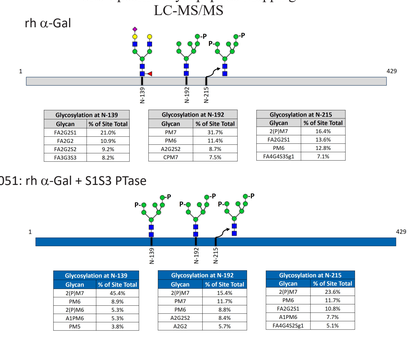
α-Galactosidase (M051) CI-MPR interaction and affinity is dramatically improved when co-expressed with S1S3 PTase

- α-Galactosidase (α-Gal) is a lysosomal enzyme that hydrolyses globotriaosylceramide (GL-3) in kidney and other cells
- It is a 60 kDa glycoprotein homodimer with 3 N-linked glycosylation sites
- Fabry Disease is caused by reduced or absent α-Gal activity resulting in GL3 accumulation in the kidney, heart and skin.

Fluorescently Labeled Glycan Chromatography



Site-Specific Glycopeptide Mapping LC-MS/MS



Glycosylation at N-139	
Glycan	% of Site Total
A2G2S1	21.0%
FA2G2	10.9%
FA2G2S2	9.2%
FA3G3S3	8.2%

Glycosylation at N-192	
Glycan	% of Site Total
PM7	31.7%
PM6	11.4%
A2G2S2	8.7%
CPM7	7.5%

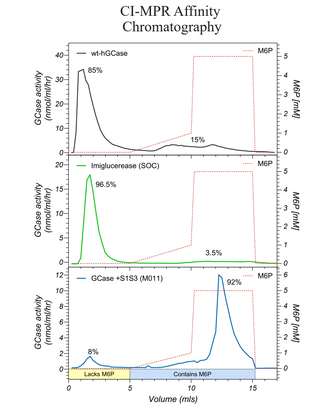
Glycosylation at N-215	
Glycan	% of Site Total
2(P)M7	16.4%
FA2G2S1	13.6%
PM6	12.8%
FA4G4S3Sg1	7.1%

Glycosylation at N-139	
Glycan	% of Site Total
2(P)M7	45.4%
PM6	8.9%
2(P)M6	5.3%
A1PM6	5.3%
PM5	3.8%

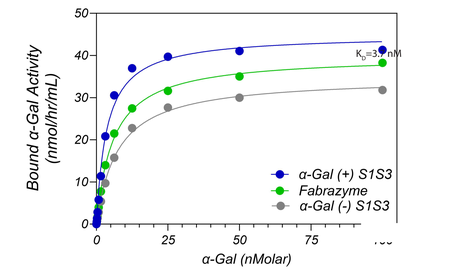
Glycosylation at N-192	
Glycan	% of Site Total
2(P)M7	15.4%
PM7	11.7%
PM6	8.8%
A2G2S2	8.4%
A2G2	5.7%

Glycosylation at N-215	
Glycan	% of Site Total
2(P)M7	23.6%
PM6	11.7%
FA2G2S1	10.8%
A1PM6	7.7%
FA4G4S3Sg1	5.1%

CI-MPR Affinity Chromatography



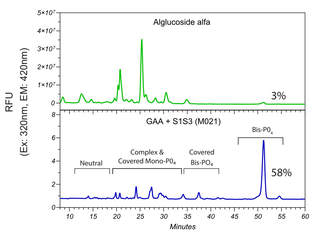
CI-MPR Binding Affinity



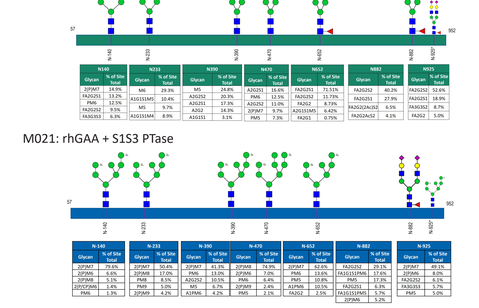
Acid-α-glucosidase (M021) CI-MPR interaction and affinity is dramatically improved when co-expressed with S1S3 PTase

- Acid-α-glucosidase (GAA) is a lysosomal enzyme required to breakdown glycogen in cardiac and skeletal muscle
- It is a 110 kDa glycoprotein with 7 N-linked glycosylation sites (7th site occupied ~ 40%-50% of the time)
- Pompe Disease is caused by reduced or absent GAA activity resulting in glycogen accumulation in the lysosome and autophagic dysfunction

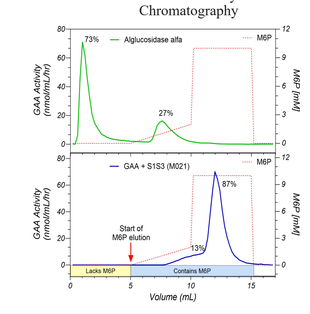
Fluorescently Labeled Glycan Chromatography



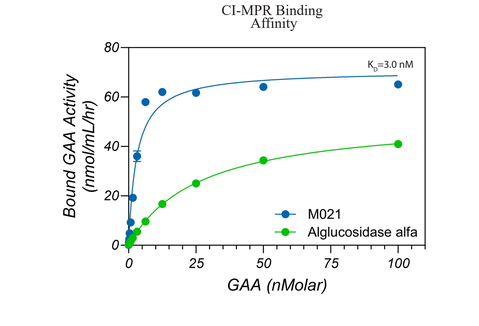
Site-Specific Glycopeptide Mapping LC-MS/MS



CI-MPR Affinity Chromatography



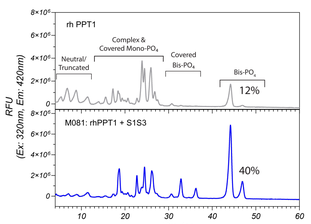
CI-MPR Binding Affinity



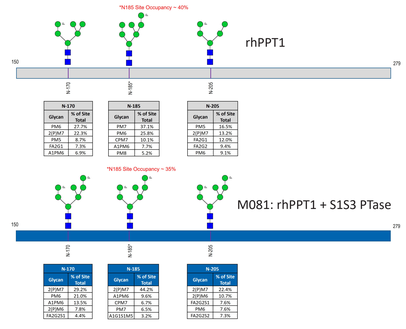
Palmitoyl-protein thioesterase 1 (M081) CI-MPR interaction and affinity is improved when co-expressed with S1S3 PTase

- Palmitoyl-protein thioesterase 1 (PPT1) is a lysosomal enzyme that removes thioester-linked fatty acyl groups during catabolism of lipid-modified proteins
- It is a 32.7 kDa glycoprotein with 2 N-linked glycosylation sites
- Batten CLN1 is caused by diminished or missing PPT1 Activity in the CNS

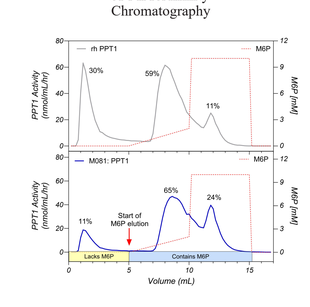
Fluorescently Labeled Glycan Chromatography



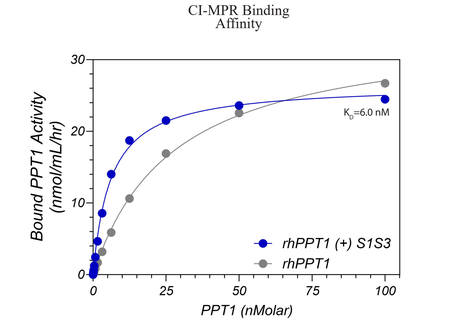
Site-Specific Glycopeptide Mapping LC-MS/MS



CI-MPR Affinity Chromatography



CI-MPR Binding Affinity



Conclusions

- S1S3 PTase efficiently and reliably phosphorylates soluble lysosomal enzymes including those that are typically poorly phosphorylated like GAA and GCase and improves phosphorylation of enzymes considered efficiently phosphorylated.
- For the 4 enzymes evaluated, all had improvements in amount mono- and bis-phosphorylated glycans, leading to nanomolar affinity for CI-MPR
- N-linked glycan processing fundamentally changes with the co-expression of S1S3 PTase
- The improvement in phosphorylation translates to better efficacy *in vivo* for all 4 enzymes by either ERT or GTx. Data not shown, available upon request