Essentials of Glycobiology

Lecture 20

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The "P-type" Lectins and the Trafficking of Lysosomal Enzymes

Subcellular Trafficking Pathways for Glycoproteins

- Other soluble glycoproteins
- Lysosomal enzymes

TRANSLATION
N-GLYOSYLATION

Rough Endoplasmic Reticulum
Intermediate Compartment
Golgi Stacks
Trans Golgi Network
Early Endosome
Late Endosome
Lysosome
Secretory Granule

Lysosomal enzymes
Other soluble glycoproteins
1960s: Exploration of Human Genetic "Storage disorders"
- Failure of intracellular lysosomal degradation of cellular components, which therefore accumulate in the lysosomes.
- Some patients accumulated "mucopolysaccharides" (now called glycosaminoglycans)
- These could be metabolically labelled in cultured fibroblasts by inorganic $[^{35}S]$sulfate (Elizabeth Neufeld & co-workers)
- This $[^{35}S]$sulfate accumulation corrected by co-cultivating abnormal cells with normal fibroblasts (or with cells from patients with a different clinical phenotype).

High-Uptake and Low-uptake Forms of Lysosomal Enzymes
- Soluble "corrective factors" turned out to be different lysosomal enzymes deficient in the patients with different diseases, and being secreted by the normal cells in small amounts
- Enzymes found to exist in two forms: a "high-uptake" form that could correct deficient cells, and a "low-uptake" form that was inactive.
- Direct-binding studies showed saturable, high-affinity receptors for the "high-uptake" lysosomal enzymes
- "High uptake" property could be destroyed by periodate treatment - predicting that this marker contained carbohydrate
I-Cell Disease
“Inclusion Cell Disease”

Fibroblasts

I-Cell Disease

- Fibroblasts from a human genetic disease with prominent "inclusion bodies" in cells ("I-cell disease") lack not one, but almost all lysosomal enzymes.
- In I-cells, all the enzymes are actually being made, but are almost completely secreted into the medium.
- Hickman and Neufeld: I-cells could take up the "high-uptake" enzymes from normal cells, but the enzymes secreted by I-cells not taken up by other cells.
I-Cell Disease

- **Hypothesis:** I-cell disease resulted from a failure to add a “common recognition marker” present on all lysosomal enzymes.

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**Major Steps in the Biosynthesis of N-glycans on Glycoproteins (including Lysosomal Enzymes):**

- **Endo-beta-N-acetyl-glucosaminidase H (Endo-H)**
- **High mannose-type glycans**
- **Complex-type glycans**
- **Hybrid-type glycans**
Structural Nature of the “High-Uptake” Marker

- Uptake of "high-uptake" lysosomal enzymes was specifically blocked by mannose 6-phosphate (M6P) and its stereoisomer fructose-1-phosphate.

- Millimolar concentrations were required, but similar concentrations of other sugars and sugar phosphates had no comparable effect.

- Since Man residues occur on high mannose-type N-glycans, it was predicted that these might be phosphorylated specifically on lysosomal enzymes.

- Confirmed by alkaline phosphatase treatment, which abolished "high-uptake" activity, and by tunicamycin treatment, which blocked N-glycosylation, and caused secretion of lysosomal enzymes from cells.

- M6P directly shown to be present in "high-uptake" forms of lysosomal enzymes and on EndoH-sensitive N-glycans from these enzymes.
Endo-H sensitive N-glycans of Lysosomal Enzymes Contain “Blocked” Phosphate residues

\[ X -P- \text{Man-(N-glycan)-Lysosomal enzyme} \]

\[ \downarrow \text{Endo H} \]

\[ X -P- \text{Man-(N-glycan)} \]

\[ \downarrow \text{Mild Acid} \]

\[ X + P- \text{Man-(N-glycan)} \]

\[ \downarrow \text{Alkaline Phosphatase} \]

\[ P + \text{Man-(N-glycan)} \]

\[ X = \text{GlcNAc!} \]

Enzymatic Steps in the Biosynthesis of the High-uptake Marker

\[ \text{Uridine-P-}^{32}\text{P-}\alpha[6-^3\text{H}]\text{GlcNAc + Man}\alpha1-(\text{N-glycan})-\text{Lysosomal enzyme} \]

\[ \downarrow \text{“Phosphotransferase”} \]

\[ \text{Uridine-P + [6-^3\text{H}]GlcNAc}\alpha1.^{32}\text{P-6-Man}\alpha1-(\text{N-glycan})-\text{Lysosomal enzyme} \]

\[ \downarrow \text{“Uncovering Enzyme”} \]

\[ [6-^3\text{H}]\text{GlcNAc} + ^{32}\text{P-6-Man}\alpha1-(\text{N-glycan})-\text{Lysosomal enzyme} \]

\[ \downarrow \text{Phosphatase} \]

\[ ^{32}\text{P} + \text{Man}\alpha1-(\text{N-glycan})-\text{Lysosomal enzyme} \]
Mannose 6-phosphate pathway for trafficking of lysosomal enzymes

**ENDOPLASMIC RETICULUM**

- Mannose
- N-linked sugar chain

**LYSOSOMAL ENZYME**

**DEFECT IN I-CELL DISEASE**

**MANNOSE-6-P-GlcNAc**

**PHOSPHODIESTER GLYCOSIDASE**

**MANNOSE-6-P**

**MANNOSE-6-P RECEPTOR(s)**

**GOLGI APPARATUS**

**ENDOSOMAL COMPARTMENT**

**LOW pH**

**LYSOSOME**

**ACID PHOSPHATASE**

**Pseudo-Hurler Polydystrophy**

(Mucolipidosis III)

A Milder version of I-cell Disease
Nature of the Defect in a Variant form of Mucolipidosis III

Failure to Recognize Lysosomal Enzymes as Special Substrates?

Structural Basis for Recognition of Lysosomal Enzymes by GlcNAc Phosphotransferase

- Not explained by similarities in primary polypeptide sequences of lysosomal enzymes.
- Denatured lysosomal enzymes lose their specialized acceptor activity.
- Features of secondary or tertiary structure are crucial.
- Sequence "swapping" between cathepsin D (M6P+) and pepsinogen (M6P-)
  - scattered basic residues critical (particularly lysines)
  - two regions of the cathepsin D amino lobe are involved
  - these cooperate with a recognition element in the carboxyl lobe
Structural Basis for Recognition of Lysosomal Enzymes by GlcNAc Phosphotransferase

- Studies with other enzymes confirm the general model: scattered basic residues + adjacent surface loops
- How does catalytic reach of the GlcNAc-phosphotransferase extend to widely spaced N-glycans on a lysosomal hydrolase target?
- Different N-glycans on the same enzyme get different degrees of phosphorylation, based on how far away they are from the recognition patch(es)

Purification of UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) 1996

- Catalyzes initial step in the synthesis of the mannose 6-phosphate determinant
- Partially purified by chromatography and used to generate a panel of murine monoclonal antibodies
- Monoclonal antibody PT18 coupled to a solid support and used to immunopurify the enzyme ~480,000-fold to apparent homogeneity
Purification of UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) 1996

- Subunit structure: a 540,000-Da complex composed of disulfide-linked homodimers of 166,000- and 51,000-Da subunits and two identical, noncovalently associated 56,000-Da subunits
- Properties essentially same as those originally described for impure enzyme
- Human cDNA and genomic clones reported in 2000

Nature of the Defect in the Variant form of ML-III

![Diagram showing differences in enzyme structure between normal and mutant forms](image-url)
N-Acetylglucosamine-1-phosphodiester alpha-N-Acetylglucosaminidase
(“Uncovering Enzyme” or Phosphodiester alpha-GlcNAcase)

- Catalyzes second step in the synthesis of the mannose 6-phosphate determinant of lysosomal enzymes
- Partially purified preparation used to generate a panel of murine monoclonal antibodies.
- Monoclonal antibody UC1 coupled to a solid support and used to immunopurify the bovine liver enzyme ~670,000-fold in two steps to apparent homogeneity

N-Acetylglucosamine-1-phosphodiester alpha-N-Acetylglucosaminidase
(“Uncovering Enzyme” or Phosphodiester alpha-GlcNAcase)

- The purified enzyme has similar properties to the original one
- Subunit structure: a 272,000-Da complex of four identical 68,000-Da glycoprotein subunits arranged as two disulfide-linked homodimers - a type I membrane-spanning glycoprotein with amino terminus in lumen of Golgi apparatus
- Human cDNA and mouse genomic DNA clones isolated in 1999
Isolation of the Mannose-6-Phosphate Receptors (M6PRs, P-type lectins)

1. **Ca++/Mg++**
   - Apply to Affinity Column Under physiological conditions

2. Non-ionic Detergent Extract
   - Cell/Tissue with Binding Sites for M6P ligands

3. **Wash well**
   - With buffer

4. **Elute**
   - With?

5. **1 mM Glc6P**
   - & discard

6. **1 mM Man6P**

7. **Dialyze**

8. **Reapply To column**

9. **Reproduce Elution specificity**

10. **SDS-PAGE**

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The Mannose 6-Phosphate Receptors (“P-type Lectins”)

- **Lysosomal Enzyme**
- **IGF-II**
- **M6P**
- **CD-MPR**
- **CT-MPR**
Ribbon diagram of the CD-MPR (Roberts et al., *Cell* 93:639-648, 1998)

**Biosynthesis of Phosphorylated N-glycans**

1 = Golgi Mannosidase I  
2 = GlcNAc Phosphotransferase  
3 = GlcNAc Transferase I  
4 = Phosphodiester glycosidase  
5 = Galactosyltransferase  
6 = Sialyltransferase(s)

Complex and hybrid-type glycans

BINDING TO MPRs
Comparison of The Mannose 6-Phosphate Receptors

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>CATION-INDEPENDENT (LARGE) RECEPTOR</th>
<th>CATION DEPENDENT (SMALL) RECEPTOR</th>
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<tr>
<td>Topology Type I Membrane glycoprotein</td>
<td>Type I Membrane glycoprotein</td>
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</tr>
<tr>
<td>Subunit MW (SDS-PAGE)</td>
<td>300-250 kDa</td>
<td>45 kDa</td>
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<tr>
<td>Core Polypeptide MW</td>
<td>275 kDa</td>
<td>30 kDa</td>
</tr>
<tr>
<td>Optimal pH for binding</td>
<td>6.0-7.0</td>
<td>6.0-6.5</td>
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<tr>
<td>Cation dependence for Binding</td>
<td>No</td>
<td>Mn⁺⁺ &gt; Mg⁺⁺ = Ca⁺⁺</td>
</tr>
<tr>
<td>Domain Structure</td>
<td>15 homologous repeating units of ~145 AA each</td>
<td>Single 155 AA unit homologous to repeating units of large receptor</td>
</tr>
<tr>
<td>Native Oligomeric State</td>
<td>Monomer</td>
<td>Dimer or tetramer</td>
</tr>
<tr>
<td>Stoichiometry of Binding to M6P</td>
<td>Two per Monomer</td>
<td>One per monomer</td>
</tr>
<tr>
<td>Kd for glycan with 2 M6P units</td>
<td>2X10⁻⁷ M</td>
<td>2X10⁻⁵ M</td>
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<tr>
<td>Binding of Other Ligands</td>
<td></td>
<td></td>
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<tr>
<td>- Methyl-Phosphomannose</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>- Insulin-like Growth factor II</td>
<td>Yes (no in chicken/Xenopus)</td>
<td>No</td>
</tr>
<tr>
<td>- Retinoic Acid</td>
<td>Yes</td>
<td>?</td>
</tr>
<tr>
<td>- Urokinase-type plasminogen activator receptor (uPAr)</td>
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<td></td>
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<tr>
<td>Role in biosynthetic pathway</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Role in endocytic pathway</td>
<td>Yes</td>
<td>(?) except at high density</td>
</tr>
</tbody>
</table>

Pathways for the Trafficking of P-type lectins

- Other soluble glycoproteins
- Lysosomal enzymes
- Clathrin-Coated Vesicles
- Secretory Granule
- Early Endosome
- Late Endosome
- Lysosome
Genetic Defects in the Mannose 6-Phosphate receptors

- Targeted disruption of CD-MPR gene in mice: normal or only slightly elevated levels of lysosomal enzymes in circulation, and otherwise normal phenotype.

- However: thymocytes from homozygous CD-MPR null mice or primary cultures of their fibroblasts show clear increase in lysosomal enzymes secretion

- Other glycan-specific endocytotic receptors (mannose-specific receptor of macrophages or asialoglycoprotein receptor of hepatocytes) provide in vivo compensation?

Genetic Defects in the Mannose 6-Phosphate receptors

- Mouse Cl-MPR is part of the naturally occurring Tme locus, a maternally imprinted region of chromosome 17 (i.e. expressed only from the maternal chromosome). Mice that inherit a deleted Tme locus from their mother die at day 15 of gestation.

- Lethality due to lack of Cl-MPR - proven by induced disruption of gene. Maternal inheritance of null allele generally lethal by birth and mutants about 30% larger in size.

- Size phenotype probably caused by excess IGF-II, because introduction of an IGF-II null allele rescued the mutant mice. Mutant mice also have organ and skeletal abnormalities
Genetic Defects in the Mannose 6-Phosphate receptors

- Fibroblasts were prepared from embryos that lack one or both receptors.
- Fibroblasts lacking only one receptor showed a partial impairment in enzyme sorting.
- Fibroblasts lacking both receptors show a massive missorting of multiple lysosomal enzymes and accumulated undigested material in their endocytic compartments.
- Thus, both receptors are required for efficient intracellular targeting of enzymes.

Genetic Defects in the Mannose 6-Phosphate receptors

- Comparison of phosphorylated proteins secreted by different cell types indicates that the two receptors interact preferentially with different subgroups of hydrolases.
- This is confirmed by in vitro studies using different enzymes and cell types.
- Heterogeneity of phosphomannosyl recognition marker within a single enzyme and amongst different enzymes explains evolution of two MPRs with complementary binding properties.
- Together with factors such as the number, compartmental localization, properties and availability of receptors, the endosomal pH, and concentration of divalent cations, there is much flexibility in this trafficking mechanism.
Evolutionary origins of the MPR system

- Lysosomal enzymes are successfully targeted in Saccharomyces, Trypanosoma and Dictyostelium, without any identifiable MPRs.

- D. discoideum produces a methyl-phosphomannose sequence on some lysosomal enzymes that can be recognized by the mammalian CI-MPR (not the CD-MPR). There is also a GlcNAc phosphotransferase that recognizes \( \alpha 1-2 \) linked Man residues, but it is not specific for lysosomal enzymes.

- Acanthamoeba produces a phosphotransferase that does show specific recognition of mammalian lysosomal enzymes.

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Evolutionary origins of the MPR system

- Although some of these organisms show evidence for an “uncovering” enzyme, no definable MPR has been found.

- A CI-MPR receptor was recently identified in a mollusc.

- The evolutionary divergence point at which the complete MPR system came into being has yet to be definitively identified.
Alternate Pathways for Trafficking of Lysosomal Enzymes

- In I-cell disease, some cells and tissues (e.g. liver, B-lymphoblast lines and circulating granulocytes) have essentially normal levels of lysosomal enzymes.

- Two soluble lysosomal enzymes, acid phosphatase and β-glucocerebrosidase are not at all affected in their distribution even in I-cell disease fibroblasts.

- Acid phosphatase begins life as a membrane-bound protein, and once in the lysosome, it is proteolytically cleaved to generate the mature soluble form.

- Glucocerebrosidase is soluble, but membrane associated, does not show phosphorylation, and is targeted to lysosomes independent of this pathway.

- Is the M6P pathway for trafficking of lysosomal enzymes a specialized form of targeting, superimposed upon some other basic mechanisms that remain undefined?

- **Note:** Integral membrane proteins of the lysosome such the lysosomal membrane proteins do not require the phosphomannosyl recognition pathway for trafficking to lysosomes. They utilize motifs in their cytosolic tails similar to those of the MPRs.
Mannose 6-phosphate on Non-lysosomal Proteins

- Hydrolytic enzymes which seem to take a mainly secretory route
  - Uteroferrin: failure of removal of the blocking GlcNAc residues?
  - DNAase I: native level of phosphorylation appears to be very low?
- TGF-beta growth factor precursor (lost in the mature form).
  - Man 6P may serve to target precursor to an acidic compartment for activation in the intact cell.

Mannose 6-phosphate on Non-lysosomal Proteins

- Renin precursor: uptake and activation?
- Proliferin: affects growth factor function?
- Leukemia Inhibitory Factor (glycosylated form)
- Thyroglobulin: targets protein for degradation & thyroid hormone release?
- Herpes Zoster and Varicella virus glycoprotein
  - M6P on complex-type N-glycans (?different pathway)
CI-MPR as a Tumor Suppressor Gene?

- Loss of heterozygosity at CI-MPR locus seen in >50% of dysplastic liver lesions and hepatocellular carcinomas associated with the high risk factors (hepatitis virus infection & liver cirrhosis).
- Mutations in the remaining allele detected in ~50% of these tumors, which seemed to develop from clonal expansions of phenotypically normal, CI-MPR-mutated hepatocytes.
- Thus, the CI-MPR may function as a liver tumor-suppressor gene.
- Note: CI-MPR can regulate distribution of some factors associated with cancer, including proteases and growth factors.

QUESTIONS?