**BIOCHEMISTRY**

**Structure of the murine lysosomal multienzyme complex core**

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The enzymes β-galactosidase (GLB1) and neuraminidase 1 (NEU1; sialidase 1) participate in the degradation of glycoproteins and glycolipids in the lysosome. To remain active and stable, they associate with PPCA [protective protein cathepsin A (CTSA)] into a high–molecular weight lysosomal multienzyme complex (LMC), of which several forms exist. Genetic defects in these three proteins cause the lysosomal storage diseases GM1-gangliosidosis/mucopolysaccharidosis IV type B, sialidosis, and galactosialidosis, respectively. To better understand the interactions between these enzymes, we determined the three-dimensional structure of the murine LMC core. This 0.8-MDa complex is composed of three GLB1 dimers and three CTSA dimers, adopting a triangular architecture maintained through six copies of a unique GLB1-CTSA polar interface. Mutations in this contact surface that occur in GM1-gangliosidosis prevent formation of the LMC in vitro. These findings may facilitate development of therapies for lysosomal storage disorders.

**INTRODUCTION**

Lysosomes are major sites of cellular catabolism where macromolecules including proteins, polysaccharides, and lipids are degraded. These processes are carried out by dozens of specific hydrolytic enzymes often acting in succession. Genetic defects in these proteins result in accumulation of undegraded intermediates and are one cause of lysosomal storage diseases (1). Two of these enzymes are acid β-galactosidase (GLB1) (2) and neuraminidase 1 (NEU1; sialidase 1) (3). These two glycosidases cleave terminal galactose and sialic acid residues, respectively, from glycoprotein and possibly glycolipid substrates. Mutations in GLB1 cause GM1-gangliosidosis/mucopolysaccharidosis IV type B (4), whereas defects in NEU1 cause sialidosis (5).

One common feature of many lysosomal enzymes is the requirement to undergo activation in lysosomes to prevent detrimental activity in the endoplasmic reticulum (ER) and Golgi during their folding and transport (6). These proteins must also remain stable against proteolysis in this degradative environment. GLB1 and NEU1 achieve this by associating with a third enzyme, PPCA [protective protein cathepsin A (CTSA)] (7). These three hydrolases form a high–molecular weight lysosomal multienzyme complex (LMC) (8–10). CTSA greatly extends the half-life of GLB1 (11, 12) and is critical for the transport, activation, and stability of NEU1 (13–16). Its importance is reflected in the disorder galactosialidosis, where mutations in CTSA disrupt the LMC and lead to secondary deficiency of GLB1 and NEU1 (7).

In addition to their catabolic roles, these three enzymes are found on the cell surface in a related assembly, the elastin receptor complex (ERC), which is involved in extracellular matrix maintenance (17, 18). The ERC participates in elastic fiber formation via binding of its GLB1 subunit [an isoform thereof named elastin-binding protein (EBP)] to elastin precursors and assisting their transport out of the cell (19). The ERC also serves as receptor for elastin degradation products (20). This interaction is an emerging antitumor target, as these degradative peptides influence multiple cellular processes (17). The NEU1 subunit of the ERC also participates in elastic fiber formation (21), modulates proliferative and immune signaling by desialylating cell surface receptors (22, 23), and is a novel therapeutic target in tumorigenesis (24).

Possible treatments for lysosomal storage diseases include pharmacological chaperone therapy (25) and enzyme replacement therapy (ERT). The latter approach is in preclinical stages for GLB1 for GM1-gangliosidosis (12, 26). Despite the isolated structures of GLB1 (27) and CTSA (28, 29) having been determined, ERT development has faced issues due to the multifaceted interplay of enzymes within the LMC (12). Although this complex was discovered 40 years ago (7), unexpectedly, little is known about its architecture and specific composition, or that of its cell surface counterpart, the ERC.

Here, we report the structure of the LMC core determined by cryo–electron microscopy (EM). This 0.8-MDa complex is composed of three GLB1 dimers and three CTSA dimers connected through six copies of a unique GLB1-CTSA polar interface. Three GM1-gangliosidosis/mucopolysaccharidosis IV type B mutations that occur at this contact surface disrupt their association in vitro. The relationship between complex formation, enzymatic activity, and proteolytic processing is rationalized, and implications for the larger, NEU1-containing assembly, as well as for the cell surface ERC, are discussed.

**RESULTS**

**The core LMC is composed of three CTSA dimers and three GLB1 dimers in a triangular architecture**

Two main forms of the LMC have been characterized: a 0.7-MDa (estimated) assembly of multiple CTSA and GLB1 subunits and a low-abundance 1.3-MDa complex that also includes NEU1 and possibly N-acetylgalactosamine-6-sulfate sulfatase (9, 30). Murine and human GLB1 and CTSA were recombinantly expressed in insect cells and purified. The “core” LMC—the focus of this study—was formed by incubating CTSA with GLB1 at pH 4.5, and...
the three-dimensional structure was determined by cryo-EM to a resolution of 4.6 Å, which was increased to 3.7 Å by density modification (figs. S1 and S2 and table S1). This level of detail allows visualization of large features such as Asn-linked glycans (fig. S3C) and many bulky side chains (fig. S3D), but the orientations of most residues were modeled approximately, facilitated by the crystal structures of human CTSA (28) and GLB1 (27).

The stoichiometry of the core LMC had not been definitively established (31–34). The structure revealed a 0.8-MDa assembly of six CTSA and six GLB1 subunits adopting a triangular arrangement, with three GLB1 dimers as sides and three CTSA dimers as vertices (Fig. 1). The complex exhibits threefold symmetry with dimensions of 220 Å by 130 Å. Both isolated GLB1 at acidic pH (31, 35–37) and isolated CTSA (16, 32–34) dimerize. The GLB1 and CTSA homodimers within the LMC are identical to the ones observed in their respective crystal structures (fig. S3, A and B) (27–29), and there are no substantial conformational differences.

Both enzymes undergo proteolytic processing at acidic pH in lysosomes (28, 29, 38, 39), with the mature form consisting of a large and small subunit in each case. The cryo-EM map displays weak density for the GLB1 segment where processing occurs (39), suggesting flexibility; this portion could not be modeled confidently. The CTSA excision peptide (29) is present in the current structure; both precursor and mature CTSA are able to form the LMC (14, 34).

**The core LMC is stabilized by six copies of a unique GLB1-CTSA polar interface**

The arrangement of subunits within the LMC was unknown, although it had been the subject of modeling studies (40). Hydrophobic interactions were proposed to maintain this complex based on its resistance to high salt concentrations (31). GLB1 consists of a TIM barrel catalytic domain flanked on one side by two β domains (27), whereas CTSA is composed of a catalytic core domain and a cap domain insertion (28). In the core LMC, each CTSA molecule contacts a single GLB1 chain and vice versa, resulting in six copies of a single type of GLB1-CTSA interface (Figs. 1B and 2A). This interaction involves two helices and a loop from the GLB1 catalytic domain (residues 198 to 260) and a loop in the CTSA catalytic domain (residues 29 to 43). This 600-Å² polar interface is mediated by up to eight hydrogen bonds and three salt bridges and has almost no hydrophobic contacts (Fig. 2, B and C). In comparison, the interaction area within the GLB1 dimer is 900 Å² and 1800 Å² within the CTSA dimer—mostly hydrophobic in both cases.

The resolution of the structure and the map quality allowed only approximate fitting of the orientations of many side chains (Fig. 3B), and their orientations may be biased by the structures of the human homologs used as reference during structure refinement. Nevertheless, the salt bridge–forming residues appear central to the interface, especially Arg102 from GLB1 and Lys43 from CTSA; both protrude into a groove on the surface of the opposite protein (Fig. 3A). The high sequence identity between the murine and human homologs (79 and 88% for GLB1 and CTSA, respectively) and conservation of the relevant residues in mammals (Fig. 2C) facilitated modeling of the human LMC based on the murine structure (fig. S4). A few human-specific potential contacts were identified, mostly on the interface periphery. The core salt bridges Arg201-Glu35, Asp198-Arg48, and Glu259-Lys43 remained conserved.

Additional interactions between LMC subunits could be mediated by Asn-linked glycans, possibly Asn127 from CTSA or Asn248 from GLB1 (Fig. 2D). Although these moieties are mostly disordered in the cryo-EM map, the glycans on the insect cell–produced recombinant proteins in this study are shorter than their mammalian versions. The larger physiologic glycans could extend further across the complex and hypothetically contribute to its stabilization.

**Fig. 1. LMC structure.** (A) Domain organization of GLB1 and CTSA, numbered according to the human sequences. The propeptides (PP) excised from mature CTSA (29) and GLB1 (54) are colored in gray. The maturation cleavage site within GLB1 is also indicated (39). SP, signal peptide. The GLB1 segment not visible in the structure is represented by a horizontal gray line. (B) Cryo-EM structure of the murine core LMC, with GLB1 dimers in lime and green and CTSA precursor dimers in yellow and orange. Three orientations are displayed. Catalytic residues of both enzymes are marked by pink spheres. Asparagine-linked glycans are depicted as white sticks. Gray arrows indicate viewing directions. Density maps are shown in figs. S2 and S3.
LMC interface mutants prevent formation of the complex and increase GLB1 susceptibility to proteolysis in vitro

The protective protein CTSA prevents excessive proteolytic degradation of GLB1 in lysosomes (7, 41), greatly extending its half-life (11, 12) via formation of the LMC (38). Genetic defects in GLB1 cause GM1-gangliosidosis/mucopolysaccharidosis IV type B (4), where stability or enzymatic activity of GLB1 is directly affected. A structural analysis of this enzyme classified mutations found in GM1-gangliosidosis and mucopolysaccharidosis IV type B according to their predicted effect on the protein (27). Notably, the substitutions Arg201 Cys, Arg201 His, and Asp198 Tyr (42) are located on the surface of the enzyme, and their impact could not be rationalized; for instance, Arg201 Cys exhibits normal specific activity (25). These two residues participate in the GLB1-CTSA interface within the LMC (Fig. 3A), and their disruption could destabilize the complex. We introduced these mutations into recombinant human GLB1 (fig. S5, A and B) and assessed their ability to associate with CTSA in vitro by size exclusion chromatography. Substitutions of Arg201 fully prevented formation of the LMC, and Asp198 Tyr had a partial effect (Fig. 3C).

No known galactosialidosis mutations occur in CTSA at its interaction surface with GLB1. To further validate this interface, we introduced artificial substitutions of Lys43, a residue establishing multiple contacts with GLB1 (Fig. 3A). Two other CTSA residues at the interface were also replaced by tryptophan to sterically block association with GLB1 (fig. S5, A and B). As expected, each of these substitutions fully or partially abrogated complex formation in vitro (Fig. 3C). The ability of these mutants to protect GLB1 from proteolytic degradation in vitro was then evaluated, mirroring the physiological role of CTSA within the LMC in lysosomes. GLB1 was susceptible to proteolysis at acidic pH by pepsin and cathepsin D, and addition of wild-type CTSA had a protective effect, whereas substitutions of Lys43 and of other interface residues decreased the capacity of CTSA to prevent degradation of GLB1 (Fig. 3D and fig. S5, C and D).

The influence of the LMC on the catalytic activity of GLB1 was also assayed in vitro. Inclusion of CTSA did not significantly increase degradation of an artificial small molecule galactosidase substrate (fig. S5E), in line with previous reports (32, 36, 43), supporting a protective but not activating role for CTSA with respect to GLB1.

DISCUSSION

Although the LMC was discovered 40 years ago (7), much uncertainty remains regarding its composition and properties. The current results shed some light on several outstanding questions.
Processing and enzymatic activity of the LMC components

Both GLB1 and CTSA undergo proteolytic processing in lysosomes (38, 39). Removal of an excision peptide frees the active site and enables the carboxypeptidase activity of CTSA (29), which is distinct from its protective role (44). This segment is located far from the GLB1-CTSA interface (Fig. 4A), explaining how both its pre-cursor and mature forms can participate in the LMC (14, 34). For the same reason, both free and GLB1-bound CTSA should be able to undergo activation. GLB1 is also processed, although this does not affect its enzymatic activity (45). Similarly, its proteolysis site is far from the LMC interface (Fig. 4A), suggesting that complex formation and processing events are independent (14).

There are no major conformational changes in the LMC relative to the structures of its isolated components (fig. S3A). The GLB1
and proteolytic stability (13, 14), activation (1), and GLB1 associates with its large subunit (Fig. 2C). NEU1 contacts the small subunit of CTSA bridged by a NEU1 dimer (8) via two copies of a CTSA-NEU1 interface (36) so a large assembly composed of two copies of the core LMC (15), and GLB1 associates with its large subunit (Fig. 2C). The lack of effect of complex formation on enzymatic activity (fig. S5E) (36) is in blue. (B) Interface residues of the human LMC model from fig. S4A that are missing in EBP are in blue.

Evidence for an alternative model of NEU1 multimerization has recently emerged, in which GLB1 is not required to associate with NEU1. The absence of GLB1 does not decrease NEU1 activity (8, 52) and may in fact increase it, whereas GLB1 supplementation in mice or overexpression causes NEU1 deficiency (12). It was proposed that GLB1 and NEU1 actually compete for binding to CTSA (12), precluding a complex of all three enzymes. This model is supported by observations of CTSA-NEU1 heterodimers that form upon combining CTSA (homodimer) with NEU1 (oligomer) (16) and the existence of higher-order complexes of both proteins (15, 30). The core LMC structure reported here is also partially compatible with this model; one of its six GLB1-CTSA interfaces could be replaced by a CTSA-NEU1 contact. Structural characterization of NEU1 and of its association with CTSA will be required to clarify the interactions between these three enzymes.

**Elastin receptor complex**

In addition to their lysosomal catabolic functions, GLB1 and NEU1 are also found on the cell surface, where they play a role in extracellular matrix formation, specifically in elastic fiber assembly (21, 53). A catalytically inactive isoform of GLB1 called EBP (54–56) serves as a chaperone for tropoelastin (the elastic fiber precursor) during its transport from the ER to the cell surface (19, 57). There, NEU1 removes sialic acid residues from extracellular matrix glycoproteins and enables tropoelastin polymerization (21). This version of the LMC contains CTSA as well (21) and is called the ERC, as it is also a sensor of peptides derived from elastin degradation (58).

The ERC has not been structurally characterized, and the structures of its NEU1 and EBP subunits are unknown. Alternative splicing of GLB1 to EBP results in replacement of a 162-residue portion of the catalytic domain by a 31–amino acid segment (54). Unexpectedly, the EBP isoform lacks almost all the residues of the GLB1-CTSA interface from the core LMC (Fig. 4C). The architecture of the ERC is therefore likely quite different from the core LMC. Further studies are needed to understand the interplay between these three enzymes in lysosomes and at the cell surface to facilitate their targeting in pathologies involving the extracellular matrix as well as their applications in replacement therapy for genetic disorders.

**MATERIALS AND METHODS**

**Protein expression and purification**

Recombinant GLB1 and CTSA were expressed as secreted proteins in S9 insect cells infected with baculovirus. The endogenous signal peptide was replaced by the melittin signal peptide MKFLVNYVALVFYMVYISYIYA followed by a hexahistidine tag DRHHHHHHHGS. Constructs encompassed residues 27 to 677 of human GLB1 (UniProt: P16278 with the polymorphism Ser532Gly), residues 28 to 647 of murine GLB1 (UniProt: P23780 with the variants Arg468Gln, Asn517Asp, and Glu534Gly), residues 29 to 480 of human CTSA (UniProt: P10619), and residues 24 to 474 of murine CTSA (UniProt: P16675). The following surface mutations were introduced with the aim of improving protein production: Cys32Ala in human GLB1 and Cys32Ala in murine CTSA. These residues are poorly conserved in mammals. The mutant proteins were used for all structural and biochemical analyses. The enzymes were isolated from expression culture medium by immobilized metal affinity chromatography, purified by size exclusion chromatography in buffer
[10 mM tris-HCl (pH 7.5) and 100 mM NaCl], concentrated to 10 mg/ml, and flash-frozen.

Cryo–electron microscopy
Concentrated murine GLB1 and CTSA were mixed in a 1:2 molar ratio—the initially presumed stoichiometry of the complex—and diluted to 0.5 mg/ml total with buffer [50 mM sodium acetate (pH 4.5) and 50 mM NaCl]. After 1 hour at 4°C, 4 μl of sample was applied to a glow-discharged nanofabricated gold-coated copper-rhodium grid with 2.5-μm holes (59). The grid was frozen in liquid ethane at liquid nitrogen temperature using a Vitrobot Mark IV (FEI) and operating at 120 kV. Images were collected at a nominal magnification of 57,000 using a charge-coupled detector camera (BM-Ceta) with a magnified pixel size of 2.45 Å. Data were collected on a Titan Krios G3 electron microscope with a Falcon 4 detector using EPU 2 (Thermo Fisher Scientific) (table S1). The sample exhibited preferential orientation in the thin layer of vitreous ice (first three classes in fig. S1B), so a 40° tilted dataset was collected in addition to the nontilted one. Optimal ice thickness was found in the center of each hole, so only one image per hole was recorded.

Image processing and structure determination
Both datasets were combined. Data were processed with cryo-SPARC v2 (60), with patch motion correction and patch contrast transfer function (CTF) estimation. Images with large areas of crystalline ice or poor CTF estimation resolution were discarded. Particles were picked using templates created from a low-resolution three-dimensional (3D) reconstruction generated from data collected on the 120-kV microscope; the original particles had been selected by the template-free blob picker in cryoSPARC. The particle box size was 320 pixels. After initial 2D classification where ice blobs, false positives, and free CTSA dimers were rejected, 34,133 particles underwent further classification to discard low-resolution and partially broken complexes—dissociation was sometimes observed at one of the GLB1-CTSA interfaces. Ab initio reconstruction was carried out with the remaining 22,537 particles, followed by 3D classification into two classes. The best class, comprising 17,591 final particles, was first refined without imposed symmetry, and after visual inspection, D3 symmetry was applied. Default refinement settings were selected, except “Minimize over per-particle scale” was enabled. The resolution was estimated using the gold standard Fourier shell correlation with a 0.143 cutoff (61) and high-resolution noise substitution (62). The map was sharpened with auto_sharpen (63) in Phenix 1.18.2 (64) without an input model. Alternatively, model-free density modification was carried out with resolve_cryo_em (65) in Phenix. The murine LMC model was built manually and real space—refined in Coot (66) starting from the crystal structures of human GLB1 (27) and CTSA precursor (28), refined against the density-modified map with real_space_refine (67), and validated with MolProbity (68) in Phenix. Refinement settings included secondary structure, Ramachandran and reference model restraints, as well as symmetry constraints; the restraints weight was set to 6. The resolution limit was 4.59 Å for all steps in Phenix. Data processing and structure determination statistics are presented in table S1. Structural images were generated with University of California San Francisco ChimeraX (69) and PyMOL (The PyMOL Molecular Graphics System, version 2.4, Schrödinger, LLC). Sequence logos were generated with WebLogo (70), and alignments were performed with MUSCLE (71).

Complex formation assay
Human GLB1 (59 μg) was incubated with human CTSA (41 μg) in binding buffer [500 μl; 25 mM sodium acetate (pH 4.5) and 500 mM NaCl] for at least 1 hour at 4°C, amounting to 1.6 μM of each protein. A high NaCl concentration was required to prevent the proteins from binding to the size exclusion chromatography resin that occurred at 100 mM NaCl at acidic pH. The complex was analyzed on a 24-ml Superose 6 Increase size exclusion chromatography column (GE Healthcare Life Sciences) at 0.75 ml/min at 4°C.

Proteolytic stability
Human GLB1 at 0.23 mg/ml (3.1 μM) was preincubated with human CTSA at 0.33 mg/ml (twofold molar excess) in buffer [50 mM sodium acetate (pH 4.5) and 100 or 500 mM NaCl] for 1 hour at 4°C. One volume of ortho-nitrophenyl-β-D-galactopyranoside (20 mM; in assay buffer) was added and incubated for 5 min at 22°C. Twenty volumes of 100 mM NaOH were added to stop the reaction, followed by absorbance measurement of o-nitrophenol at 410 nm. The reaction rate was linear with respect to GLB1 concentration.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/20/eabf4155/DC1

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