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Oligomerization and carbohydrate binding of glucan phosphatases

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ABSTRACT

Glucan phosphatases are a unique subset of the phosphatase family that bind to and dephosphorylate carbohydrate substrates. Family members are found in diverse organisms ranging from single-cell red algae to humans. The nature of their functional oligomerization has been a source of considerable debate. We demonstrate that the human laforin protein behaves aberrantly when subjected to Size Exclusion Chromotography (SEC) analysis due to interaction with the carbohydrate-based matrix. This interaction complicates the analysis of laforin human disease mutations. Herein, we show that SEC with Multi-Angle static Light Scattering (SEC-MALS) provides a method to robustly define the oligomerization state of laforin and laforin variants. We further analyzed glucan phosphatases from photosynthetic organisms to define if this interaction was characteristic of all glucan phosphatases. Starch EXcess-four (SEX4) from green plants was found to lack significant interaction with the matrix and instead exists as a monomer. Conversely, Cm-laforin, from red algae, exists as a monomer in solution while still exhibiting significant interaction with the matrix. These data demonstrate a range of oligomerization behaviors of members of the glucan phosphatase family, and establish SEC-MALS as a robust methodology to quantify and compare oligomerization states between different proteins and protein variants in this family.

1. Introduction

Glucan phosphatases are members of the Dual Specificity Phosphatase (DSP) family [1]. Their substrates are phosphorylated carbohydrates, and they function as key regulators of carbohydrate metabolism. Glucan phosphatases from various kingdoms of life have been identified based on the presence of both DSP and Carbohydrate Binding Module (CBM) domains [2]. The human glucan phosphatase laforin is a critical regulator of glycogen metabolism, and mutations in the laforin gene result in Lafora's Disease (LD), a devastating, fatal, autosomal recessive juvenile epilepsy [3]. Plant glucan phosphatases are critical for starch metabolism, and loss results in a Starch EXcess phenotype [4].

Glucan phosphatases from different kingdoms act on different carbohydrate substrates, and possess varied domain architecture (Fig. 1). Vertebrate laforin, typified by the human protein, Hs-laforin possesses an N-terminal CBM20 carbohydrate binding domain and C-terminal DSP domain. Green plant glucan phosphatases, typified by the *Arabidopsis thaliana* (At) SEX4, possess an N-terminal chloroplast Targeting Peptide (cTP), DSP, CBM48, and plant-specific C-terminus (CT) [5]. Green plants additionally contain two other glucan phosphatases, Like-SEX4 (LSF)1 and LSF2 [6]. Red algae, typified by the *Cyanidioschyzon merolae* (Cm)-laforin, contain a variable N-terminal region that is predicted to be intrinsically disordered, followed by two CBM20, and a C-terminal DSP domain. Because of the domain identity and organization, it has been hypothesized that vertebrate and red algae proteins are most similar [2]. However, since they act on different substrates and contain different domain combinations and structure, the similarity and difference between the function of glucan phosphatases from different kingdoms remains to be determined.

Laforin is a multi-domain and multi-functional protein. Previous studies have identified key aspects of the functionality of laforin, including carbohydrate binding, protein interactions, catalytic activity, and dimerization. There has been debate regarding the equilibrium, regulation, and function of different laforin oligomers [7–9]. Recent work demonstrated the structural basis for stable dimerization in solution, mediated by the DSP domain, and showed that human disease mutations were found at the dimer interface and are deleterious to protein oligomerization [10]. This result motivated studies of laforin dimerization, and development of methods to reliably determine the oligomerization state of patient mutations. We demonstrate that SEC-MALS allows reliable determination of laforin dimerization and is well suited for assessing the oligomerization of human patient mutations, critically decoupling carbohydrate binding from oligomerization. The

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Abbreviations

SEC	Size Exclusion Chromotography
SEC-MALS	Size Exclusion Chromatography with Multi-Angle static Light Scattering
SEX4	Starch EXcess-four
DSP	Dual Specificity Phosphatase
CBM	Carbohydrate Binding Module
LD	Lafora's Disease
At	*Arabidopsis thaliana*
cTP	chloroplast Targeting Peptide
CT	C-terminus
LSF	Like-SEX4
Cm	*Cyanidioschyzon merolae*
WT	Wild type
IMAC	Immobilized Metal Affinity Chromatography
BME	2-mercaptoethanol
DTT	Dithiothreitol
RI	Refractive Index
SAXS	Small Angle X-ray Scattering

oligomerization of glucan phosphatases from other kingdoms of life was also analyzed, and revealed that glucan phosphatases from other kingdoms exist in monomeric form. These data establish a reliable method to assess the differing oligomerization behaviors of glucan phosphatases and variants from different kingdoms.

2. Materials and methods

2.1. Protein expression and purification

Wild type (WT) and mutant human laforin [10], SEX4 [5], and SEX4ΔCBM [11] were expressed and purified as previously reported. Cm-laforin (domains 123, residues 157–532 C480S) and sub-domain constructs, CBM linker (domains 12l, residues 157–379), CBM (domains 12, residues 157–356), and DSP (domains 3, residues 379–532 C480S) were expressed from pET28b in *E. coli* strain Rosetta-2 (DE3) (Millipore Sigma, Burlington, MA). Cells were grown in Terrific Broth to an OD_{600} 1.5, cold-shocked for 15 min, induced with 1 mM IPTG, and grown overnight at 16 °C. Cell pellets were harvested and frozen at −20 °C. Cells were lysed using lysozyme and sonication. Initial purification was accomplished by Immobilized Metal Affinity Chromatography (IMAC) with HIS-Select HF resin (Millipore Sigma, St. Louis, MO). Protein was loaded and washed in 20 mM Tris, 100 mM NaCl, 5 mM 2-mercaptoethanol (BME), pH 8.0 and eluted with 300 mM Imidazole, 100 mM NaCl, 5 mM BME, pH 8.0. Subsequently, proteins were purified by preparative size exclusion using an AKTA pure with Superdex 75 Hi-Load 16/60 column (GE Healthcare Life Sciences, Marlborough, MA) equilibrated and run in SEC buffer [20 mM Tris, 100 mM NaCl, 2 mM Dithiothreitol (DTT), pH 7.5]. Additional SEC analysis was performed using a silica-based Bio-Select SEC 125–5 column (Bio-Rad, Hercules, CA) run in SEC buffer.

2.2. SEC-MALS

Proteins were run on an AKTA pure with inline Superdex 75 Increase 10/300 column (GE Healthcare Life Sciences), miniDAWN TREOS, and Optilab T-rEX (Wyatt Technologies, Santa Barbara, CA).

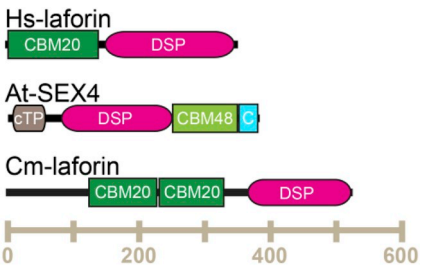

Fig. 1. Domain schematic of glucan phosphatases. Different domain type and organization are characteristic of the enzymes from different kingdoms, with amino acid numbers indicated along the horizontal axis. Domain types include: Carbohydrate Binding Module (CBM), Dual Specificity Phosphatase (DSP), chloroplast Targeting Peptide (cTP), and C-terminus (C).

The column was equilibrated and run in SEC buffer, 500 μL of 1–2 mg/mL protein was loaded, and the column run at 0.5 mL/min. Addition of 100 mM maltose in the running buffer was used to determine the effect of laforin retention. The apparent molecular weight from SEC analysis was calculated by fitting the elution volume of the protein standards Bovine albumin, Ovalbumin, Carbonic Anhydrase and Lysozyme. Light scattering data were processed using ASTRA (Wyatt Technologies). Molecular weight was determined by analyzing peaks at half height using Refractive Index (RI) as the concentration source. Further analysis and graphics were prepared using Prism (Graphpad Software, La Jolla, CA).

3. Results and discussion

3.1. Analysis of human laforin

One of the key features of laforin is its ability to oligomerize, but there is considerable debate as to the nature and effect of patient mutations on oligomerization. We first analyzed WT human laforin using SEC-MALS. We found that laforin elutes as a single species. When calibrated versus proteins of known molecular weight, SEC analysis indicates that it elutes at a volume consistent with monomeric protein. However, SEC-MALS indicates that it exists as a dimer in solution (Fig. 2A, Table 1), consistent with our previous determination by analytical ultracentrifugation and Small Angle X-ray Scattering (SAXS). To test whether the divergent retention of wild-type laforin was due to residual matrix interactions, since Superdex is a carbohydrate-based size exclusion matrix, we tested the behavior of wild-type laforin in the presence and absence of maltose. Indeed, we find that there is a significant decrease in elution volume in the presence of maltose and corresponding increase in molecular weight (Fig. 2A, Table 1). This suggests that previous assessments of the oligomerization of laforin by SEC were ambiguous due to combined effects from both oligomerization and laforin interactions with the carbohydrate-based resin, providing an explanation for previous discrepant findings.

These results indicate that SEC-MALS is a reliable method to directly determine the oligomerization state of laforin patient mutations, without effects from other functionality of the protein. We next analyzed two patient mutations, W32G and K87T, both located at the carbohydrate binding interface of the CBM domain which have dramatically reduced carbohydrate binding. In both cases the proteins eluted as a single peak, but at different volume than wild-type protein strongly suggesting that residual interaction of the CBM domain with the column matrix is responsible for the aberrant elution behavior (Fig. 2B). SEC analysis suggested an apparent molecular weight between monomer and dimer. However, analysis by SEC-MALS indicated that the proteins exist as stable dimers indicating a robust ability to decouple changes in oligomerization and carbohydrate binding (Table 1). We next assessed the C266S mutation, located in the laforin DSP-domain active site, which is catalytically inactive. In this case, the mutant protein behaved as wild-type protein and SEC-MALS analysis demonstrated the protein exists as a stable dimer (Fig. 2B, Table 1). These data indicate that laforin oligomerization and carbohydrate