Insights into the mechanism of polysaccharide dephosphorylation by a glucan phosphatase

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Members of the protein tyrosine phosphatase (PTP) family usually catalyze the dephosphorylation of protein substrates. However, some enzymes from a subclass of the PTPs, the dual specificity phosphatases (DSPs), dephosphorylate nonproteinaceous substrates such as lipids and polysaccharides (1–3). In PNAS, Vander Kooi et al. (4) describe the crystal structure of the glucan phosphatase, starch excess 4 (SEX4), providing the first insight into the structural basis of complex carbohydrate dephosphorylation by this emerging family of phosphatases.

Glycogen and amylopectin, the main constituent of starch, are branched polymers of glucose that share a basic purpose—both serve as osmotically neutral glucose stores, synthesized under conditions of nutritional plenty for utilization in times of metabolic need. As examples, liver glycogen is synthesized in the fed state and then mobilized to maintain blood glucose levels during fasting (5); leaves of green plants synthesize starch in the daytime by using energy from photosynthesis that is then used to generate maltose and glucose for energy production during darkness (6). They share a fundamental chemical structure—both contain glucose units connected by α-1,4-glycosidic linkages with branch points formed by α-1,6-glycosidic linkages (Fig. 1*A*). Lastly, and most relevant to our discussion here, both polysaccharides contain small amounts of covalently linked phosphate, one phosphate per 500–1,500 glucoses in glycogen (3, 7) and one per 150–300 glucoses in amylopectin (8).

Recent research has focused on the covalent phosphate in the two polysaccharides. In amylopectin, the phosphate is present as C3 and C6 phosphomonoesters and is introduced by the concerted action of two dikinase enzymes, glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) (9) (Fig. 1*D*). Phosphate is released by SEX4, a member of the dual specificity protein phosphatase family that additionally contains a C-terminal carbohydrate binding module (CBM) of the CBM48 subtype (Fig. 1*B*). Much less is known about the metabolism of the covalent phosphate in glycogen. Animals lack dikinases equivalent to GWD and PWD, and the origin of the phosphate is only now being worked out. Also, the chemical nature of the phosphate linkage to glycogen is uncertain. However, its hydrolysis is catalyzed by a DSP, called laforin (3), that has important similarities to SEX4. The laforin DSP domain is also flanked by a CBM, in this case an N-terminal CBM20 subtype (Fig. 1*B*). Laforin is primarily restricted to vertebrates, although it is found in certain protists that produce a scarcely branched glucose polymer called floridean starch (10). SEX4 and laforin may represent an example of convergent evolution (11) and, indeed, laforin can partially rescue the phenotype of plants defective in SEX4 (10).

What is the function of the phosphate in amylopectin and glycogen? The best evidence to date comes from genetics. SEX4 is named for the phenotype resulting from its mutation, starch excess, and is involved in the catabolism of starch (12). The branching in amylopectin is discontinuous, leading to clusters of aligned polyglucose helices that form insoluble crystalline lamellae that are metabolically inert (Fig. 1*C*) (13). The prevailing view is that phosphorylation of glucose residues in these crystalline regions by the GWD and PWD dikinases disrupts the structure to allow hydration, thereby allowing access to degradative enzymes, β-amylases (BAM1 and BAM3) and isoamylase (ISA3), that generate oligosaccharides (12) (Fig. 1*D*). In the absence of SEX4 to hydrolyze the C3 and C6 monoesters, starch degradation is impaired and phospho-oligosaccharides accumulate (12). Thus, the function of amylopectin phosphorylation is in a transient step of normal starch degradation.

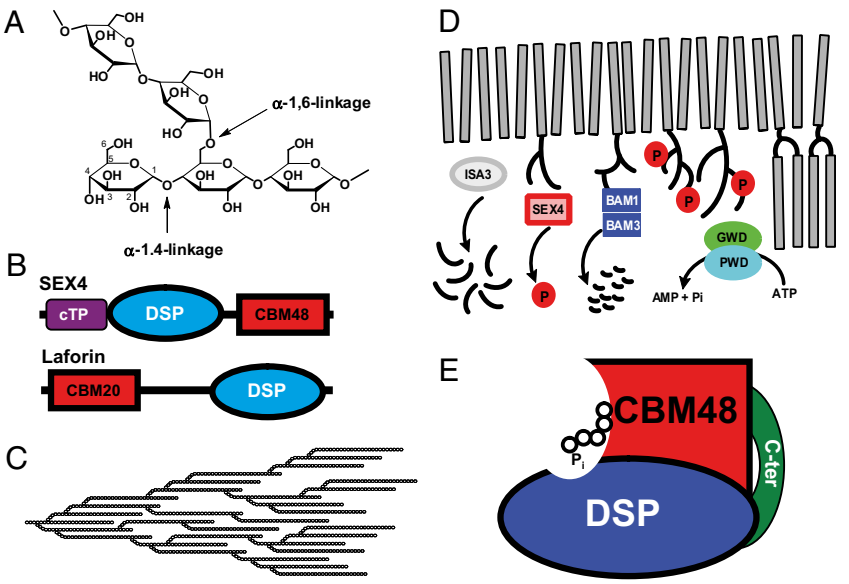

Fig. 1. Amylopectin structure, catabolism, and dephosphorylation. (*A*) The main polymerizing α-1,4- and branching α-1,6-linkages shared by amylopectin and glycogen. (*B*) Comparison of the architectures of the SEX4 and laforin phosphatases. cTP, chloroplast targeting peptide. (*C*) Representation of the branching structure of amylopectin. Clustering of the branch points is thought to result in parallel polyglucose helices that adopt a crystalline-like structure. (*D*) Starch degradation. Phosphorylation of starch by GWD and PWD disrupts the packing of amylopectin double helices (gray cylinders), allowing the release of maltose and oligosaccharides by the β-amylases, BAM1 and BAM3, and the isoamylase, ISA3. SEX4 dephosphorylates C3 and C6 monophosphates, allowing efficient starch degradation and preventing the accumulation of phosphorylated oligosaccharides. (*E*) Cartoon of the SEX4 protein structure highlighting two of its most interesting features, namely that the CBM and the DSP interact to form a compact protein and that both domains contribute to the formation of a unique catalytic pocket that can accommodate a chain of multiple glucose residues. C-ter, C-terminal domain.

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Less is known about the function, if any, of glycogen phosphorylation, but mutations in *Epm2a*, the gene encoding laforin, lead to Lafora disease, a fatal teenage onset progressive myoclonus epilepsy (14, 15). The disease is associated with the formation, in many tissues, of Lafora bodies, insoluble deposits containing poorly branched glycogen that resembles amylopectin. These deposits in humans contain increased phosphate (16) and, in mice lacking laforin, increased glycogen phosphate accompanies the development of abnormalities in glycogen properties and structure as the mice age (7). Even if glycogen phosphate has an evolved function, excessive phosphate is deleterious and must be removed by laforin in what could be considered a repair mechanism.

Therefore, the biological functions of SEX4 and laforin may be distinct, but the catalytic tasks that they have evolved to perform, of vital importance in different ways to their respective organisms, clearly have much in common. The structure of SEX4 reported by Vander Kooi et al. (4) is a critical first step toward understanding the mechanism and substrate selectivity of polysaccharide dephosphorylation by this new class of carbohydrate phosphatases. From the initial bioinformatic analyses of SEX4 and laforin that revealed the presence of DSP and CBM domains, one simplistic expectation was that the CBM would sequester the phosphatase to the polysaccharide and increase the local phosphatase concentration to facilitate dephosphorylation reactions. Some support for this view came from the observation that disabling the laforin CBM eliminated its ability to dephosphorylate glycogen but not the low molecular weight artificial substrate *p*-nitrophenylphosphate (3). However, fusion of laforin's CBM to the DSP VHR did not result in an active polysaccharide phosphatase (2), indicating that the DSP domain contributes to substrate specificity.

Both results are accommodated by the SEX4 crystal structure (4), a striking feature of which is that multiple contacts connect the DSP and CBM domains to form a compact protein. Furthermore, both domains contribute to creating a unique catalytic site, 21 Å wide, that is needed to cope with the phospho-oligosaccharide substrate (Fig. 1*E*). Vander Kooi et al. (4) propose that an oligosaccharide chain of five to six glucose residues would be needed to span from the CBM to the site of catalysis in the DSP. It is still possible that the CBM could provide a necessary initial contact, thus setting up oligosaccharides of variable structures to position their C3 or C6 phosphates for hydrolysis by the DSP domain. Most DSPs, including SEX4, contain an Asp residue in the highly conserved D-loop that acts as a general acid catalyst in the formation of a phospho-enzyme intermediate and then as a general base activating a molecule of water to hydrolyze the thiophosphate bond (17). Nearly all DSPs contain a short chain hydrophilic residue directly C-terminal to the Asp. However, SEX4 contains a Phe (F167) at this position that is located between the Asp (D166) and a critical Trp residue in the CBM involved in carbohydrate binding (W278). Mutation of F167 decreased the glucan phosphatase activity of SEX4, consistent with the authors' prediction that F167 is critical to couple polysaccharide binding and catalysis by orienting the substrate directly into the extended catalytic cleft.

SEX4 and laforin may represent an example of convergent evolution.

There are several other structural features of SEX4 that distinguish it from DSPs that have protein substrates. First, the active site variable insert, a conserved region involved in positioning the Arg in the active site to interact with the phosphate of the substrate (17), is significantly longer in SEX4 and consists of two α-helices, whereas the variable insert usually lacks secondary structure. Second, the AYLM motif, which is highly conserved in DSPs and forms part of the extended catalytic HCX_5R motif, is not conserved in SEX4. Instead, this region contains a TYMF motif in which the Phe interacts with the C-terminal helix and provides an important contact that helps stabilize the protein.

It will be interesting in the future to uncover more details of the binding of phospho-oligosaccharide substrates. An interesting unanswered question relates to the degree of flexibility required of the catalytic pocket—how heterogeneous are the natural substrates? Is the relationship of the phosphate to branch points fixed or variable, because of the specificity of the dikinases and/or other amylopectin metabolizing enzymes? Also, of course, it will be fascinating to compare the active site of SEX4 with that of laforin, when that structure becomes available.

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