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Review

Glycogen phosphorylation and Lafora disease

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ABSTRACT

Covalent phosphorylation of glycogen, first described 35 years ago, was put on firm ground through the work of the Whelan laboratory in the 1990s. But glycogen phosphorylation lay fallow until interest was rekindled in the mid 2000s by the finding that it could be removed by a glycogen-binding phosphatase, laforin, and that mutations in laforin cause a fatal teenage-onset epilepsy, called Lafora disease. Glycogen phosphorylation is due to phosphomonoesters at C2, C3 and C6 of glucose residues. Phosphate is rare, ranging from 1:500 to 1:5000 phosphates/glucose depending on the glycogen source. The mechanisms of glycogen phosphorylation remain under investigation but one hypothesis to explain C2 and perhaps C3 phosphate is that it results from a rare side reaction of the normal synthetic enzyme glycogen synthase. Lafora disease is likely caused by over-accumulation of abnormal glycogen in insoluble deposits termed Lafora bodies in neurons. The abnormality in the glycogen correlates with elevated phosphorylation (at C2, C3 and C6), reduced branching, insolubility and an enhanced tendency to aggregate and become insoluble. Hyperphosphorylation of glycogen is emerging as an important feature of this deadly childhood disease

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1. Introduction

Bill Whelan's career has spanned many decades and many research interests, though the latter, were unified by his passion for carbohydrates, and storage polymers like glycogen and starch in particular. My own work since the 1980s has sometimes overlapped with Bill's, especially in the area of glycogenin, whose discovery ranks among Bill's most notable achievements and which is addressed in other articles of this issue. Here, though, I will focus on another, seemingly esoteric feature of glycogen metabolism on which Bill had made important observations in the early 1990s, namely the covalent phosphorylation of glycogen. His work lay relatively undisturbed in the literature until quite

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recently when it began to be appreciated that excessive phosphorylation of glycogen was associated with, and might even cause, Lafora disease, a rare but deadly teenage-onset form of epilepsy. And so our work once again bumped into key findings made by the Whelan laboratory.

2. Glycogen phosphorylation

2.1. History

Phosphate is a relatively abundant and ubiquitous biomolecule and, as such, is a potential and frequent contaminant of many purified cellular constituents. Such was true for purified glycogen and, indeed, in early studies, low phosphate content was viewed as a positive indicator of the purity of the glycogen (Mordoh et al., 1966; Northcote, 1954; Wanson and Drochmans, 1968). Already, however, plant starch, which resembles glycogen in being a branched polymer of glucose, was recognized as containing covalent phosphate (Hizukuri et al., 1970). The first convincing report of stably bound phosphate in glycogen was by Fontana (1980) in 1980. Whelan and colleagues followed up with a series of studies (Kirkman and Whelan, 1990; Lomako et al., 1993, 1994) in which they confirmed the presence of covalent phosphate, suggested that it was present as a C6 phosphomonester or as a C1–C6 phosphodiester, and proposed the existence of an enzyme, separate from glycogen synthase, that could transfer a glucose-phosphate moiety from UDP-glucose to form the phosphodiester linkage.

2.2. Chemistry

The chemistry of the attachment of phosphate to glycogen was re-visited recently, in large part because of the discovery of a mechanism to release it from glycogen by a phosphatase called laforin (Tagliabracci et al., 2007). Laforin is the product of the *EPM2A* gene, one of two genes whose mutation causes Lafora disease (Chan et al., 2003; Minassian et al., 1998; Serratosa et al., 1999). In *Epm2a*–/– mice (hereafter designated laforin–/– mice), a rodent model of the human disease, the phosphorylation of glycogen increases up to ten-fold as the animals age (Tagliabracci et al., 2007) and the excess phosphate appears to contribute significantly to the pathology of the disease (see Section 2.5). The stoichiometry of glycogen phosphorylation is low, in the range of 1 phosphate per 500 glucoses to 1 phosphate per several thousand glucoses, depending on the source of glycogen and the particular study. Because of the scarcity of the phosphate, we hydrolyzed rabbit muscle glycogen with glucosidases and enriched for negatively charged species by anion exchange chromatography (Tagliabracci et al., 2011). Analysis of the bound material by mass spectrometry indicated a mixture of compounds dominated by species with masses corresponding to hexose oligosaccharides plus a phosphate, consistent with the purification of phospho-oligosaccharides of glucose. Despite the size-heterogeneity, analysis of this sample by NMR gave clear signals indicative of glucose phospho-monoesters at the C2 and the C3 atoms but did not observe either C6 phosphate or phosphodiesters. A subsequent study (Nitschke et al., 2013), using similar methods for purification of phospho-oligosaccharides, detected C2, C3 as well as C6 phosphate by NMR, but, as in our work, did not observe phosphodiesters (Tagliabracci et al., 2011). In addition, Nitschke et al. (2013) measured glucose-6-P directly in hydrolysates of glycogen from mouse and rabbit sources. The two papers presented conflicting views as to the presence of C6 phosphate in glycogen; we therefore pursued the question further, testing the hypothesis that methods of purifying glycogen might affect the phosphorylation pattern (DePaoli-Roach et al., 2015). We analyzed rabbit muscle glycogen isolated by our previous, relatively gentle procedure, in fact following more or less the old Whelan protocol used to preserve glycogenin attached to the glycogen (Kennedy et al., 1985). In addition, we used a more extreme procedure in which powdered frozen muscle is treated first with boiling KOH. Results from either protocol were similar and in both cases we now detected C6 phosphorylation by NMR analyses (DePaoli-Roach et al., 2015). We also developed a sensitive assay for glucose-6-P in glycogen hydrolysates and were able to quantitate C6 phosphorylation in glycogen samples that, combined with analysis of total covalent phosphate, permitted us to calculate the relative contribution of C6 and C2 + C3 phosphorylation to the total phosphate content. C6 phosphate typically makes up around 20% of the total phosphate with C2 + C3 phosphate accounting for the majority, around 80%, in either mouse or rabbit muscle glycogen samples (DePaoli-Roach et al., 2015). This phosphate distribution contrasts with amylopectin in which most phosphate is associated with C6 (Blennow et al., 2002; Stitt and Zeeman, 2012), for example ~90% in our recent study of potato amylopectin, the rest of the phosphate at C3, and little evidence for significant phosphorylation at C2 (DePaoli-Roach et al., 2015). Though the presence of phosphodiesters cannot be completely discounted, these recent studies did not find any indications for their existence.

2.3. Metabolism

The metabolism of the covalent phosphate within glycogen is not yet fully understood. The laforin phosphatase (Minassian et al., 1998; Serratosa et al., 1999) by sequence belongs to the atypical dual specificity protein phosphatase (DSP) sub-family (Alonso et al., 2004). Laforin is unique in being the only protein phosphatase in the genome that contains a built-in carbohydrate binding domain (CBM20) at its N-terminus as well as a phosphatase domain. After an essentially fruitless search for protein substrates, it emerged that laforin can act on polysaccharide substrates like amylopectin (Tagliabracci et al., 2007; Worby et al., 2006) and glycogen (Tagliabracci et al., 2007). In biochemical experiments, mutation of the CBM to disable carbohydrate binding also eliminates the ability of laforin to dephosphorylate glycogen while leaving the active site capable of hydrolyzing generic substrates like *p*-nitrophenol phosphate (Tagliabracci et al., 2007). Glycogen purified from the muscle of mice lacking laforin contains elevated levels of phosphate compared with controls (Tagliabracci et al., 2007, 2008). Therefore, it is currently quite well accepted that laforin functions as a glycogen phosphatase *in vivo*. Plants contain two glycan phosphatases, SEX4 and LSF2, that