Biochemical and Biophysical Research Communications xxx (2016) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The regulatory α and β subunits of phosphorylase kinase directly interact with its substrate, glycogen phosphorylase

Jackie A. Thompson, Gerald M. Carlson*

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA

ARTICLE INFO

Article history:
Received 12 October 2016
Accepted 8 November 2016
Available online xxx

Keywords:
Crosslinking
Phosphorylase kinase
Glycogen phosphorylase
Substrate recognition
Docking sites

ABSTRACT

The selective phosphorylation of glycogen phosphorylase (GP) by its only known kinase, phosphorylase kinase (PhK), keeps glycogen catabolism tightly regulated. In addition to the obligatory interaction between the catalytic γ subunit of PhK and the phosphorylatable region of GP, previous studies have suggested additional sites of interaction between this kinase and its protein substrate. Using short chemical crosslinkers, we have identified direct interactions of GP with the large regulatory α and β subunits of PhK. These newfound interactions were found to be sensitive to ligands that bind PhK.

1. Introduction

The breakdown of glycogen is mediated by phosphorylase kinase (PhK) and glycogen phosphorylase (GP). PhK phosphorylates a single serine on GP, causing activation and subsequent phosphorolysis of glycogen to release glucose-1-phosphate [1]. PhK is also regulated by reversible phosphorylation, and both GP and PhK are also regulated by a variety of allosteric effectors [2,3]. This complex regulation of PhK and GP is consistent with their large masses. PhK is a hexadecameric complex having four copies of four different subunits (α, β, γ and δ) and a total mass of 1.3 MDa [2]. The γ subunit (44.7 kDa) is catalytic, and the remaining three subunits, α (138.4 kDa), β (125.2 kDa) and δ (16.7 kDa), are regulatory [2], with the δ subunit being a molecule of non-dissociable calmodulin. The substrate GP is a homodimer of 197 kDa that has two distinct faces: a regulatory face, where the phosphorylatable serine and allosteric binding sites reside, and a catalytic face [4].

The recognition of protein substrates by kinases can be complex. The selective phosphorylation of protein substrates depends not only on having appropriate amino acids surrounding the residue to be phosphorylated (primary structure), but also frequently on docking site interactions distinct from the phosphorylation site(s) [5]. After decades of extensive work with peptide substrates, consensus amino acid sequences preferred by most kinases are reasonably well-defined, but docking site interactions between kinases and their substrates are by comparison poorly understood [5–7]. These distinct contact points often play important roles in substrate recognition and phosphorylation [6,7].

Unlike most other kinases, PhK has only one recognized physiological target, GP. Moreover, GP is known to be phosphorylated only by PhK, making this an unusually specific kinase-substrate pair. Despite this unusual specificity and the importance of these two enzymes, little is known regarding the physical interaction between PhK and GP, and their large sizes complicate traditional binding and structural studies. We know that the N-terminus of GP, which contains its single phosphorylation site, must bind to the active site of γ to be phosphorylated, and the crystal structure of truncated γ with a peptide substrate reveals details of this binding [8]. Importantly, the only comprehensive binding studies on the interactions between PhK and GP found that GP lacking its phosphorylatable N-terminus still bound to PhK with a similar affinity as full-length GP [9], suggesting additional contact site(s). Yeast two-hybrid studies raised the possibility that the regulatory α subunit of PhK may interact with GP [10]. Calmodulin, PhK's δ subunit, has also been shown to bind an N-terminal fragment of GP [11]. Only the β subunit has not been previously implicated in the binding of GP. Here we report the use of short chemical crosslinkers to unambiguously show direct interactions between GP and the two

Abbreviations: AMP-PNP, adenylyl-imidodiphosphate, a nonhydrolyzable ATP analogue; ANB-NOS, N-5-azido-2-nitrobenzoyloxysuccinimide; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; GP, glycogen phosphorylase (nonactivated form); PhK, phosphorylase kinase; RT, room temperature; SIA, succinimidyl iodoacetate.

* Corresponding author. 3901 Rainbow Boulevard, Mailstop 3030, Kansas City, KS 66160, USA.
E-mail address: gcarlson@kumc.edu (G.M. Carlson).

http://dx.doi.org/10.1016/j.bbrc.2016.11.044
0006-291X/

large regulatory subunits of PhK, α and β.

2. Materials and methods

2.1. Enzymes

Non-activated PhK was purified from New Zealand White rabbit psoas muscle as previously described [12], dialyzed into 50 mM HEPES (pH 6.8), 0.2 mM EDTA, and 10% sucrose (w/v), and stored at −80 °C. GP was also isolated from New Zealand White rabbit muscle as described previously [13], and recrystallized with Mg^{2+} and AMP. After removal of AMP by dialysis into 10 mM HEPES (pH 6.8), GP was stored at −80 °C. The concentrations of PhK and GP were determined spectrally as previously described [12].

2.2. Chemical crosslinking

The crosslinkers 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (CAS No. 327-92-4), succinimidyl iodoacetate (SIA) (CAS No. 39028-27-8), and N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) (CAS No. 60117-35-3) were from Pierce/ThermoFisher Scientific.

To limit crosslinking, a two-step protocol was used with DFDNB and SIA in the crosslinking of GP with PhK. In step 1, GP was pre-incubated with crosslinker (135 μM DFDNB or 540 μM SIA) for 2–5 min at room temperature (RT). The pre-incubated GP was immediately diluted 10-fold into a solution containing PhK and incubated at 30 °C for 5 min. The final concentrations were: 20 mM HEPES (pH 6.8), 0.1 mM EDTA, 437 μg/mL PhK, 260 μg/mL GP, and 13.5 μM DFDNB or 54 μM SIA (carryover solvent was 0.2% DMSO or ACN). The molar ratio of PhK protomer (αβγδ) to GP monomer was 1:2, while the ratio of DFDNB and SIA to GP monomer was 5:1 and 20:1, respectively. Reaction aliquots were quenched in an equal volume of SDS buffer (125 mM Tris (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, 4% SDS, and trace Coomassie R250) and analyzed on a 4–12% linear SDS-PAGE gel.

ANB-NOS, a photo-activated crosslinker, was treated differently than DFDNB and SIA. GP was incubated with 100 μM ANB-NOS for 5 min at RT in the dark. After removal of unreacted ANB-NOS on a P10 de-salting column, the labeled GP was incubated at RT with PhK under a long-wave UV lamp (366 nm) at a distance of 1 cm for 5 min. The crosslinking reaction was quenched and analyzed as above.

When effectors were included in the DFDNB crosslinking reaction, they were added to the GP/DFDNB solution 15 s before addition of PhK. The final concentrations of Mg^{2+}, Ca^{2+}, and AMP-PNP in the second step were 4 mM, 330 μM, and 300 μM, respectively. The density of the crosslinked products was determined using ImageJ software. To ensure reproducibility, three different preparations of PhK were used, and all experiments were performed in triplicate.

2.3. Western blotting

Samples for immunodetection were transferred from SDS-PAGE gels to PVDF membranes and blocked with 5% (w/v) nonfat powdered milk in 0.14 M NaCl, 2.7 mM KCl, 6 mM P_i (pH 7.4), 0.1% Tween20, and 0.2% gelatin. The primary antibodies against GP and the α, β, and γ subunits of PhK have been previously characterized and were used as described [14–16]. Colorimetric detection of the immunoreactive bands was performed with AP-conjugated secondary antibodies from Southern Biotechnology.

3. Results and discussion

3.1. Identifying GP-PhK crosslinkers

To limit the amount of crosslinking, a two-step crosslinking approach was used to capture interactions between GP and subunits of PhK. This approach involved pre-labeling GP with a low concentration of crosslinker (step 1) immediately prior to incubation with PhK (step 2). The pre-labeling of GP preferentially captured conjugates between PhK and GP before intramolecular crosslinking within the GP dimer or PhK hexadecamer could dominate. Of seven different crosslinking reagents screened using this two-step method, four (sulfosuccinimidyl 4,4'-azipentanoate; bis(sulfosuccinimidyl)suberate; N-α-maleimidoacet-oxy-succinimide ester; and formaldehyde) failed to capture GP-PhK conjugates, but three (DFDNB, SIA, and ANB-NOS) successfully did so.

Several major products are formed from DFDNB crosslinking of GP and PhK. The two most intense crosslinked bands are intramolecular PhK products, formed with or without GP present (Fig. 1A, lane 4). One is a slower migrating α-β conjugate, and the other is an intra-subunit crosslink of β (Fig. 1B). When DFDNB, PhK and GP are present together, two new bands form below the α-β product (Fig. 1A, lane 3), the slower being a doublet that cross-reacts with anti-GP and anti-α antibodies, identifying it as a GP-α conjugate. The faster migrating band is a GP-β conjugate. There are additional PhK-GP products above the α-β band; however, these are not well-defined, and their composition is difficult to confidently identify by Western blots due to their proximity to one another. Finally, GP undergoes a small amount of inter-subunit crosslinking to form three, faint bands (Fig. 1A, lane 2).

Another crosslinking reagent, SIA, also formed GP-PhK conjugates (Fig. 2A). While GP and PhK were both independently crosslinked by SIA (Fig. 2A, lanes 1 and 3), when they were present together, three new products formed (lane 2). Western blotting identified the products as GP-α, GP-β, and a second, faster migrating GP-β ($GP\text{-}\beta_F$) conjugates (Fig. 2A, bottom panel). The GP-α product is a doublet, like the GP-α formed by DFDNB, suggesting that multiple sites are crosslinked between GP and α.

ANB-NOS, the third crosslinker that formed GP-PhK conjugates, required a different crosslinking approach than used with DFDNB and SIA. ANB-NOS is a photosensitive crosslinker, containing one functional group that remains inert until activated by UV light. Therefore, GP was labeled in the dark by the amine-selective group on ANB-NOS, and unreacted ANB-NOS was removed by gel filtration prior to incubation of the modified GP with PhK. UV light then activated the second functional group on the crosslinker, allowing it to react with bound PhK. Like SIA, ANB-NOS formed three GP-PhK conjugates (Fig. 2B), which were identified by Western blots as another GP-α doublet, and two GP-β products.

Thus, three different crosslinking reagents apparently form the same GP-PhK conjugates (Fig. 2C). In the case of the GP-α doublet and the slower migrating GP-β, these conjugates co-migrate for all three crosslinkers. The faster migrating $GP\text{-}\beta_F$ appears with SIA and ANB-NOS, but not DFDNB. The consistent formation of these products suggests that the same interfaces between GP and regions of α and β are being sampled by the different crosslinkers and represent genuine contact points between GP and PhK. Moreover, the short spacer lengths of the crosslinkers (1.5–7.7 Å) further argues for direct contact of GP with α and β. Our results are consistent with earlier predictions of direct interactions between GP and the regulatory subunits of PhK based on binding studies. GP lacking its phosphorylatable N-terminus binds to PhK in a competitive ELISA assay, but fails to bind the isolated catalytic subunit [9], suggesting that the regulatory subunits of PhK may be involved in the binding

Please cite this article in press as: J.A. Thompson, G.M. Carlson, The regulatory α and β subunits of phosphorylase kinase directly interact with its substrate, glycogen phosphorylase, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.11.044