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O-GlcNAcylation of the *Plum pox virus* capsid protein catalyzed by SECRET AGENT: characterization of O-GlcNAc sites by electron transfer dissociation mass spectrometry

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Abstract

The capsid protein of *Plum pox virus* (PPV-CP) is modified with *O*-linked β -*N*-acetylglucosamine (*O*-GlcNAc). In *Arabidopsis thaliana* this modification is made by an *O*-GlcNAc transferase named SECRET AGENT (SEC). Modification of PPV-CP by SEC is hypothesized to have a direct role in the infection process, because virus titer and rate of spread are reduced in SEC mutants. Previous studies used deletion mapping and site-directed mutagenesis to identify four *O*-GlcNAc sites on the capsid protein that are modified by *Escherichia coli*-expressed SEC. The infection process was not affected when two of these sites were mutated suggesting that *O*-GlcNAcylation of these sites does not have a significant role in the infection process or that a subset of the modifications is sufficient. Since it is possible that the mutational mapping approach missed or incorrectly identified *O*-GlcNAc sites, the modifications produced by *E. coli*-expressed SEC were characterized using mass spectrometry. *O*-GlcNAcylated peptides were enzymatically tagged with galactose, the products were enriched on immobilized *Ricinus communis* agglutinin I and sequenced by electron transfer dissociation (ETD) mass spectrometry. Five *O*-GlcNAc sites on PPV-CP were identified. Two of these sites were not identified in by the previous mutational mapping. In addition, one site previously predicted by mutation mapping was not detected, but

modification of this site was not supported when the mutation mapping was repeated. This study suggests that mapping modification sites by ETD mass spectrometry is more comprehensive and accurate than mutational mapping.

Keywords

O-GlcNAc; *Plum pox virus*; Capsid protein; *O*-GlcNAc transferase; *Arabidopsis thaliana*; SECRET AGENT; Electron transfer dissociation tandem mass spectrometry

Introduction

Genes predicted to encode *O*-GlcNAc transferases (OGTs) occur in all eukaryotes with the exception of select fungi (Butkinaree et al. 2010; Hanover et al. 2010; Olszewski et al. 2010). In addition, genes encoding enzymes with structures that are highly similar to the eukaryotic OGTs exist in many bacteria (Martinez-Fleites et al. 2010; Olszewski et al. 2010). Eukaryotic OGTs modify specific serine and/or threonine residues of both nuclear and cytoplasmic proteins. Each site is modified with a single *O*-linked β -*N*-acetylglucosamine (*O*-GlcNAc). In animals, *O*-GlcNAcylation is a reversible process that is involved in a diverse array of cellular processes (Butkinaree et al. 2010; Hanover et al. 2010).

Arabidopsis thaliana has two OGTs, SECRET AGENT (SEC) and SPINDLY (SPY) (Olszewski et al. 2010). Studies of SEC and SPY mutants have identified roles for these enzymes in diverse plant processes (Olszewski et al. 2010). SPY mutants have defects in light regulation, leaf development, flowering time, circadian rhythms, gibberellin signaling, and cytokinin signaling. Loss of SEC activity causes a reduction in the rate of leaf production while the loss of both SEC and SPY results in embryo lethality.

Only a few targets of OGTs have been identified in plants, one of which is the capsid protein of *plum pox virus* (PPV-CP) (Chen et al. 2005; Fernandez-Fernandez et al. 2002). Genetic studies suggest that *O*-GlcNAc has a role in the infection of *Arabidopsis* and that SEC, but not SPY, modifies PPV-CP (Chen et al. 2005). While PPV-CP is modified in wild-type and SPY plants, it is not modified in SEC plants. In addition, PPV titer and movement are reduced in the SEC mutant. Further evidence that SEC modifies PPV-CP in plants comes from the observation that *E. coli*-expressed SEC modifies it (Scott et al. 2006).

Deletion and site-directed mutagenesis studies using *E. coli*-expressed SEC identified T19, T24, T41, and S43 as sites of *O*-GlcNAcylation (Scott et al. 2006). Site-directed mutagenesis demonstrated that T19 and T24 are also modified in *Arabidopsis* (de Jesus Perez et al. 2006). However, mutation of T19 and T24 to alanine did not impair infection (de Jesus Perez et al. 2006), suggesting that a phenotypically 'wild-type' infection could be accomplished through modification of T41, S43 or possibly other sites. Since mapping strategies based on mutagenesis can be incomplete, we have directly mapped modifications made by *E. coli*-expressed SEC on wild-type PPV-CP using electron transfer dissociation (ETD) tandem mass spectrometry (MS/MS) (Syka et al. 2004). This method has already shown great promise for the characterization of labile post-translational modifications such as phosphorylation and *O*-GlcNAcylation on proteins (Chi et al. 2007; Wang et al. 2010).

Materials and methods

Co-expression with SEC and purification of His-tagged PPV

The plasmids and methods for expressing SEC and PPV-CP have been described previously (Scott et al. 2006). pACYC-Mal-SEC expresses maltose binding protein-tagged SEC. PP4 and PP4-2 are constructed in a pET32a backbone and express PPV-CP amino acids 1–100 and 1–64, respectively (Table 1). PP4 and PP4-2 were expressed in *E. coli* BL21-AiTM (Invitrogen Life Technologies, Carlsbad, CA) containing either pACYC184 or pACYC-Mal-SEC. For protein expression, a 500 ml culture was grown at 22°C to an OD₆₀₀ of 0.4 when expression of T7 RNA polymerase, which drives the expression of the PPV fusion protein, was induced by the addition of arabinose to 0.2% (w/v). After 1 h, PPV-CP and SEC expression was induced by the addition of isopropyl- β -D-thiogalactoside to a final concentration of 1 mM. After an additional 2 h of growth, the cells were harvested by centrifugation; the cell pellet was stored at –20°C. Cells were resuspended in 20 ml of 50 mM sodium phosphate, 500 mM sodium chloride, pH 8.0 and lysed using a French press (Milton Roy, Ivyland, PA) at 10,000 PSI. HIS-tagged PPV fusion protein was purified using the ProBond Purification System (Invitrogen Life Technologies, Carlsbad, CA) following manufacturer directions. The purified proteins were concentrated and buffer exchanged into 50 mM Tris-HCl (pH 7.5), 1 mM CaCl₂ using an Amicon Ultra-15 centrifugal filter unit (Millipore Corporation, Billerica, MA).

Detection of S-tagged PPV-CP and O-GlcNAcylated proteins

Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). S-tagged PPV fusion proteins were detected using S-protein HRP conjugate (Novagen, Madison, WI) using the manufacturers protocol. HRP was detected using Super Signal West Pico (Pierce, Rockford, IL) with exposure to X-OMATTM Blue XB-1 film (Eastman Kodak, Rochester, NY). To identify O-GlcNAc modified proteins, terminal GlcNAc moieties were labeled with [³H]galactose using galactosyl transferase (Roquemore et al. 1994), with modifications described previously (Heese-Peck et al. 1995; Kamemura et al. 2002). ³H-labeled proteins were detected by fluorography. The membrane was sprayed to saturation with EN³HANCE (Perkin-Elmer Life Science, Inc., Boston, MA), allowed to air dry and exposed to pre-flashed (Laskey and Mills 1975) BioMax XAR film (Eastman Kodak, Rochester, NY) at –80°C.

Enrichment of O-GlcNAc-modified peptides by RCA I affinity chromatography

Purified PPV-CP (0.8 mg) was reduced in 2 mM Tris(2-carboxyethyl)phosphine at room temperature for 1 h and then alkylated in 10 mM iodoacetamide in the dark at room temperature for 1.5 h. Mass Spectrometry Grade Trypsin (Promega, Madison, WI) was added to a final protease:protein ratio of 1:40 (w/w) and incubated at 37°C overnight. Tryptic peptides were desalted using a Sep-Pak C₁₈ cartridge (Waters, Milford, MA) and dried. GlcNAc groups on peptides were capped with either unlabeled or [³H]galactose using galactosyl transferase (Sigma, St. Louis, MO) as described previously (Ball et al. 2006; Vosseller et al. 2006). Tryptic peptides were desalted using a Sep-Pak C₁₈ cartridge and dried. RCA I affinity chromatography was performed by modifications to published procedures (Hayes et al. 1995; Haynes and Aebersold 2000). The sample was dissolved in 100 μ l of PBS (6.7 mM K₂HPO₄, 150 mM NaCl, 0.02% NaN₃, pH 7.4) and loaded onto a 1.2 m long RCA I agarose (Vector Laboratories, Burlingame, CA) column constructed in Teflon tubing (1.55 mm i.d. $\times$ 1.2 m long with a 0.5 μ m end frit; Chrom Tech, Apple Valley, MN). The separation was conducted using a flow rate of 50 μ l/min using a peristaltic pump at room temperature. After 3 ml of PBS flowed over the column, bound peptides were eluted with 0.2 M lactose in PBS. Peptides were detected by measuring absorbance at 280 nm; in GlcNAc-³H-Gal experiments, modified peptide content in the eluate was monitored by

[³H]galactose detection. Fractions containing *O*-GlcNAcylated peptides were pooled and dried using a Speed-Vac. The peptides were desalted using OMIX C₁₈/100 µl tips (Varian, Palo Alto, CA), dried and stored at −80°C.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis

Peptide samples were purified using a C₁₈ zip-tip (Millipore Corporation, Billerica, MA) and eluted using 60% acetonitrile, 0.1% trifluoroacetic acid. A 1.2 µl elution aliquot was mixed on target with the MALDI matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) and analyzed in reflector mode on a MALDI-TOF Bruker Reflex III (Bruker Daltonics, Bremen, Germany). Processing of the spectra and data analysis was performed with Bruker Daltonics XTOF 3.1.

LC-MS/MS analysis of CP-PPV peptides

Enriched PPV-CP tryptic peptides were reconstituted in 0.1% acetic acid and pressure loaded onto a 360 µm o.d. × 75 µm i.d. microcapillary pre-column packed with C18 (5–20 µm diameter, 120 Å) as previously described (Udeshi et al. 2008). The column was washed with 0.1% acetic acid and subsequently connected to a 360 µm o.d. × 50 µm i.d. microcapillary analytical column packed with C18 (5 µm diameter, 120 Å). The analytical column was equipped with an integrated electrospray emitter tip (Martin et al. 2000). Peptides were gradient-eluted into the mass spectrometer at a flow rate of 60 nl/min using the HPLC gradient, 0–60% solvent B in 60 min (A = 0.1 M acetic acid, B = 70% acetonitrile, 0.1 M acetic acid). High-resolution, accurate mass measurements were recorded on peptide ions using an LTQ-FTMS instrument operated at a resolving-power of 1,00,000 (at *m/z* 400). An LTQ-XL mass spectrometer was utilized to record ETD MS/MS spectra that were acquired on using the following parameters; reaction time = 100 ms, full AGC target = 2E4 ion counts, MSn AGC target = 2E4 ion counts, isolation window = 4 *m/z*, reagent AGC target = 4E5 ion counts, ETD reagent = fluoranthene, supplemental activation = enabled. All PPV-CP peptide sequences were confirmed by manual interpretation of the corresponding ETD MS/MS spectra.

Results

Mutation studies have indicated that *E. coli*-expressed SEC *O*-GlcNAcylates T19, T24, T41, and S43 of PPV-CP (Scott et al. 2006) and that T19 and T24 are modified in planta (de Jesus Perez et al. 2006). However, mutagenesis-based approaches for characterizing post-translational modifications carry the risk of perturbing the enzyme recognition site and thus missing or incorrectly identifying modification sites. Therefore, we decided to characterize *O*-GlcNAcylated sites on wild type PPV-CP using ETD tandem mass spectrometry. This strategy affords the opportunity to characterize GlcNAc-modified residues without directly affecting any protein structure-dependent cellular processes, a potential detriment to mutagenesis-based studies.

Due to the substoichiometric abundance of GlcNAc-modified peptides within living systems, the proximate elution of both GlcNAc-modified and unmodified counterpart peptides during C18 LC-MS experiments, and the limited dynamic range of such experiments, it is important to implement an enrichment procedure to maximize the chance of successful MS-based GlcNAc identifications. Therefore, we first determined if the modified form predominated when PP4-2 was co-expressed with SEC in *E. coli*. While terminal GlcNAc on PP4-2 protein was readily detectable by a radioactive GalT labeling assay (Fig. 1a), peptides with masses consistent with GlcNAc modification were not detected when trypsin digests of PP4-2 were subjected to MALDI-TOF analysis (Fig. 1b),

indicating that only a small fraction of PP4-2 was modified. This evidence pointed to the necessity of an enrichment procedure prior to successful mapping of the modification.

GlcNAcylated peptides were enriched using RCA lectin column chromatography (Hayes et al. 1995; Haynes and Aebersold 2000). When peptides modified with GlcNAc and [³H]galactose were separated using the RCA column, two classes of labeled peptides were resolved from the unlabeled peptides (Fig. 2). Migration of the first class of peptides was retarded relative to their unmodified counterparts while peptides in the second class required lactose elution. The migration of unmodified and galactosylated PP4-2 peptides on the RCA column was examined in more detail by MALDI-TOF (Fig. 3). Modified peptides were not detected in either the sample loaded onto the column or the fractions containing the bulk of the peptides. However, modified peptides were detectable and therefore successfully enriched in the fractions containing class 1 and class 2 peptides.

Sequence analysis of enriched PP4-2 by ETD MS/MS, demonstrated that residues T19, T24, T41, and T53 (Fig. 4; Table 2) are modified by SEC. Surprisingly, modification of S43, which was identified as a site that it is modified by SEC using a mutational mapping approach (Scott et al. 2006), was not detected. The mutational mapping study found that restoring S43 to a mutant PPV-CP in which all of the modifications sites are mutated to alanine was sufficient to restore weak labeling of the protein by the GalT assay, which labels modified proteins with [³H]galactose. Since the initial study was not performed using highly purified PPV-CP, a highly purified preparation of the mutant PPV-CP with S43 restored was tested for modification using the GalT assay but no evidence of modification was obtained (not shown), suggesting that S43 is not *O*-GlcNAcylated.

PP4-2, a truncated form of PPV-CP, was used in all initial mapping studies because previous studies found that all of the modifications were located in this portion of PPV-CP. Moreover, PP4-2 is more highly expressed and a better SEC substrate in *E. coli* than longer forms of the coat protein. Since the current study identified a modification at T53, which was not identified by the mutational mapping strategy, we examined a larger portion of PPV-CP (PP4) that contained the first 100 amino acids of the coat protein. PP4 was found to be modified at S65 in addition to those sites found in PP4-2.

Discussion

ETD MS/MS analysis of PPV-CP enabled identification of five *O*-GlcNAc sites resulting from modification of PP4-2 by SEC. Two of these sites, T53 and S65, were not previously identified using a mutational mapping strategy (Scott et al. 2006), which also identified S43 as a site of probable modification. However, GlcNAcylation of S43 was not supported by either the ETD MS analysis or a reexamination of a highly purified form of the mutant protein that was originally used to show modification of this site. Due to the potential shortcomings present in mutagenesis-based approaches and the fact that additional sites were identified in the current MS analysis exclusively, we are not confident that S43 is truly a target of SEC.

There are several possible explanations for the failure to detect T53 and S65 modifications using the mutational mapping approach. Deletion mapping delimited the modification sites to the 64 aa portion of PPV-CP contained in PP4-2. In the previous study, mutating seven T/S of PP4-2 to alanine completely blocked modification to PPV-CP. The modification sites were then identified by restoring individual S/T residues to determine which were sufficient for modification. This approach could have resulted in missed modification sites determined by enzyme recognition sites that are extended or consist of multiple regions, but this seems unlikely because OGT recognition sites are generally small (Chalkley et al. 2009; Vosseller

et al. 2006; Wang et al. 2010). However, there is evidence that some substrates may have a more complex interaction with the OGT. The carboxyl terminal domain of RNA polymerase II becomes a better in vitro substrate for human OGT as the size of the substrate is increased (Comer and Hart 2001). In addition, deletion of a portion of the TPR domain reduced the modification of RNA polymerase II suggesting that this substrate interacts with OGT at multiple sites. However, modification of PPV-CP increases as SEC TPRs are deleted, indicating that modification does not require interaction with most of the TPR domain (Oldenhof and Olszewski unpublished). It is also possible that the amino acid mutations cause the mutant proteins to fold into a structure that does not allow for proper interaction with OGT. It is interesting to note that T53 and S65 are the most C-terminal of the modification sites. If SEC modified sites moving from the N-terminus toward the C-terminus, mutations affecting upstream sites could block modification of downstream sites. However, this hypothesis is not supported because modification of upstream sites is not required for modification of T24 or T41 (Scott et al. 2006).

A number of *O*-GlcNAcylation sites have been identified on proteins from human and mouse. Although these modification sites do not occur within the context of a strict consensus sequence, many of the sites fall within the loose consensus of P/V–P/V–V/T/S–T/Sg–S/T–S/A/G/P–X–T–T (Chalkley et al. 2009; Vosseller et al. 2006; Wang et al. 2010). Disregarding the modified position, four positions surrounding T19 and T53 and three surrounding T24 and T31 conform to the consensus (Table 1), suggesting that modification site selection is similar for both the mammalian OGT and SEC.

The two PPV-CP sites known to be modified in plants (de Jesus Perez et al. 2006) are also modified by *E. coli*-expressed SEC, suggesting that the substrate specificity of SEC is similar in both *E. coli* and plants. Therefore, it will be important to learn if the other sites identified in this study are also modified in planta. It is interesting that PPV-CP is highly modified in plants (de Jesus Perez et al. 2006) but only weakly modified when it is co-expressed with SEC in *E. coli* (Fig. 1). These observations could indicate that plant cells contain additional proteins or cofactors needed to fully activate SEC.

Based on the described experiments, RCA chromatography effectively enriched modified peptides (Fig. 3). As has been observed with other peptides (Haynes and Aebersold 2000), the modified PPV peptides could be placed into two classes. One class consisted of a peptide with a single *O*-GlcNAc modification that demonstrated reduced mobility but was not significantly retained on the column. Peptides in the second class, which were retained on the column had at least one modification and were more hydrophobic than those in the first class.

Previous studies have shown that SEC modifies PPV-CP and that PPV infection is impaired in SEC plants suggesting that modification of PPV-CP by SEC is required for a normal infection. However, when plants were infected with a mutant virus where two of the SEC modification sites were mutated to alanine, the infection was wild-type suggesting that the remaining modification sites were sufficient for a normal infection and/or that SEC affects infection through modification of a host protein(s). This work has identified two new modification sites and shown that a previously identified modification site is likely not modified. This novel information makes it now possible to create mutant viruses to test the important role of *O*-GlcNAc modification of PPV-CP in the infection process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ball LE, Berkaw MN, Buse MG. Identification of the major site of O-linked beta-N-acetylglucosamine modification in the C terminus of insulin receptor substrate-1. *Mol Cell Proteomics*. 2006; 5:313–323. [PubMed: 16244361]
- Butkinaree C, Park K, Hart GW. O-linked beta-N-acetylglucosamine (O-GlcNAc): extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. *Biochim Biophys Acta*. 2010; 1800:96–106. [PubMed: 19647786]
- Chalkley RJ, Thalhammer A, Schoepfer R, Burlingame AL. Identification of protein O-GlcNAcylation sites using electron transfer dissociation mass spectrometry on native peptides. *Proc Natl Acad Sci USA*. 2009; 106:8894–8899. [PubMed: 19458039]
- Chen D, Juarez S, Hartweck L, Alamillo JM, Simon-Mateo C, Perez JJ, Fernandez-Fernandez MR, Olszewski NE, Garcia JA. Identification of secret agent as the O-GlcNAc transferase that participates in Plum pox virus infection. *J Virol*. 2005; 79:9381–9387. [PubMed: 16014901]
- Chi A, Huttenhower C, Geer LY, Coon JJ, Syka JE, Bai DL, Shabanowitz J, Burke DJ, Troyanskaya OG, Hunt DF. Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc Natl Acad Sci USA*. 2007; 104:2193–2198. [PubMed: 17287358]
- Comer FI, Hart GW. Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry*. 2001; 40:7845–7852. [PubMed: 11425311]
- de Jesus Perez J, Juarez S, Chen D, Scott CL, Hartweck LM, Olszewski NE, Garcia JA. Mapping of two O-GlcNAc modification sites in the capsid protein of the potyvirus Plum pox virus. *FEBS Lett*. 2006; 580:5822–5828. [PubMed: 17014851]
- Fernandez-Fernandez MR, Camafeita E, Bonay P, Mendez E, Albar JP, Garcia JA. The capsid protein of a plant single-stranded RNA virus is modified by O-linked N-acetylglucosamine. *J Biol Chem*. 2002; 277:135–140. [PubMed: 11606576]
- Hanover JA, Krause MW, Love DC. The hexosamine signaling pathway: O-GlcNAc cycling in feast or famine. *Biochim Biophys Acta*. 2010; 1800:80–95. [PubMed: 19647043]
- Hayes BK, Greis KD, Hart GW. Specific isolation of O-linked N-acetylglucosamine glycopeptides from complex mixtures. *Anal Biochem*. 1995; 228:115–122. [PubMed: 8572267]
- Haynes PA, Aebersold R. Simultaneous detection and identification of O-GlcNAc-modified glycoproteins using liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2000; 72:5402–5410. [PubMed: 11080893]
- Heese-Peck A, Cole RN, Borkhsenius ON, Hart GW, Raikhel NV. Plant nuclear pore complex proteins are modified by novel oligosaccharides with terminal N-acetylglucosamine. *Plant Cell*. 1995; 7:1459–1471. [PubMed: 8589629]
- Kamemura K, Hayes BK, Comer FI, Hart GW. Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. *J Biol Chem*. 2002; 277:19229–19235. [PubMed: 11904304]
- Laskey RA, Mills AD. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur J Biochem*. 1975; 56:335–341. [PubMed: 1175627]
- Martin SE, Shabanowitz J, Hunt DF, Marto JA. Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem*. 2000; 72:4266–4274. [PubMed: 11008759]
- Martinez-Fleites C, He Y, Davies GJ. Structural analyses of enzymes involved in the O-GlcNAc modification. *Biochim Biophys Acta*. 2010; 1800:122–133. [PubMed: 19647042]
- Olszewski NE, West CM, Sassi SO, Hartweck LM. O-GlcNAc protein modification in plants: evolution and function. *Biochim Biophys Acta*. 2010; 1800:49–56. [PubMed: 19961900]

- Roquemore E, Chou T-Y, Hart GW. Detection of O-linked N-acetylglucosamine (O-GlcNAc) on cytoplasmic and nuclear proteins. *Methods Enzymol.* 1994; 230:443–460. [PubMed: 8139512]
- Scott CL, Hartweck LM, de Jesus Perez J, Chen D, Garcia JA, Olszewski NE. SECRET AGENT, an *Arabidopsis thaliana* O-GlcNAc transferase, modifies the Plum pox virus capsid protein. *FEBS Lett.* 2006; 580:5829–5835. [PubMed: 17027982]
- Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc Natl Acad Sci USA.* 2004; 101:9528–9533. [PubMed: 15210983]
- Udeshi ND, Compton PD, Shabanowitz J, Hunt DF, Rose KL. Methods for analyzing peptides and proteins on a chromatographic timescale by electron-transfer dissociation mass spectrometry. *Nat Protoc.* 2008; 3:1709–1717. [PubMed: 18927556]
- Vosseller K, Trinidad JC, Chalkley RJ, Specht CG, Thalhammer A, Lynn AJ, Snedecor JO, Guan S, Medzihradszky KF, Maltby DA, Schoepfer R, Burlingame AL. O-linked N-acetylglucosamine proteomics of postsynaptic density preparations using lectin weak affinity chromatography and mass spectrometry. *Mol Cell Proteomics.* 2006; 5:923–934. [PubMed: 16452088]
- Wang Z, Udeshi ND, Slawson C, Compton PD, Sakabe K, Cheung WD, Shabanowitz J, Hunt DF, Hart GW. Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates cytokinesis. *Sci Signal.* 2010; 3:ra2. [PubMed: 20068230]

Fig. 1.

PP4-2 co-expressed with SEC in *E. coli* is partially modified. **a** GlcNAc on PP4-2 and PP4 was detected by labeling with [³H]galactose (*upper panel*) only from samples expressed in the presence of SEC (+). Probing with S-HRP (*lower panel*), which detects the PPV fusion protein confirmed that similar amounts of PPV-CP were present in each sample. **b** MALDI-TOF analysis of trypsinized PP4-2 after capping the GlcNAc with galactose. Each Gal-GlcNAc modification adds 365 Da of mass. The *arrows* indicate unmodified forms of peptides that were shown previously to be modified (Scott et al. 2006) and the *asterisks* indicate the predicted mass of the singly modified forms of these peptides

Fig. 2.

RCA I affinity chromatography of PP4-2 peptides. After capping *O*-GlcNAc on PP4-2 with [^3H]galactose, the protein was digested with trypsin, the resulting peptides were fractionated on RCA I column and 100 μl fractions were collected. Peptide (*filled square*) was detected by measuring OD_{280} and modified peptides were detected by monitoring [^3H]galactose (*empty square*)

Fig. 3.

RCA I affinity chromatography resolves PP4-2 trypsin fragment bearing Gal-GlcNAc from unmodified peptides. MALDI-TOF analysis of PP4-2 peptides in different RCA I column fractions from an experiment similar to that in Fig. 2; **a** fractions 17–24, **b** fractions 32–38, **c** fractions 39–45 and **d** fractions 53–60. Fractions 53–60 contain peptides that were eluted with lactose

Fig. 4. *O*-GlcNAc site mapping on PPV-CP peptide using liquid chromatography-MS/MS. ETD MS/MS spectrum recorded on $[M + 5H] + 5$ ions (m/z 1,003.3) corresponding to doubly GlcNAc–Gal modified PPV-CP peptide AMADEREDEEEVDAGKPSVVG TAPAAGTSPILQPPPPVIQPAPR, followed by supplemental activation. An ETD-enabled LTQ mass spectrometer was operated to record an ETD MS spectrum on m/z 1,003.3 in addition to one data-dependent scan after every MS1 scan. Three MS/MS scans were averaged to obtain this spectrum. Predicted c' and z'' -type ions are listed above and below the peptide sequence, respectively. Singly and doubly charged fragment ions are listed as monoisotopic and average masses, respectively. Ions observed and labeled in the spectrum are *underlined* and demonstrate that the *O*-GlcNAc moieties are located on Thr-21 and Thr-26. Ions corresponding to charge-reduced species and those resulting from neutral losses are bracketed. The *inverted triangle* represents peaks that fall within the precursor isolation window

Table 1

PPV-CP expressing constructs

Construct	PPV sequence ^a
PP4-2	ADEREDEEEVDAGKPS <u>V</u> <u>V</u> T <u>A</u> <u>P</u> AATSPILQPPPVIQPA <u>P</u> <u>R</u> <u>T</u> TASMLNPFTPATTQPATKPVSQV
PP4	ADEREDEEEVDAGKPSVVT <u>A</u> <u>P</u> AAT <u>S</u> <u>P</u> ILQPPPVIQPA <u>P</u> <u>R</u> <u>T</u> TASMLNPFT <u>P</u> <u>A</u> <u>T</u> <u>T</u> Q <u>P</u> A <u>T</u> K <u>P</u> <u>V</u> <u>S</u> <u>Q</u> <u>V</u> <u>S</u> <u>G</u> <u>P</u> <u>Q</u> <u>L</u> <u>Q</u> <u>T</u> <u>F</u> <u>G</u> <u>T</u> <u>Y</u> <u>G</u> <u>N</u> <u>E</u> <u>D</u> <u>A</u> <u>S</u> <u>P</u> <u>S</u> <u>N</u> <u>S</u> <u>N</u> <u>A</u> <u>L</u> <u>V</u> <u>N</u> <u>T</u> <u>N</u> <u>R</u> <u>D</u> <u>R</u> <u>D</u> <u>V</u> <u>D</u> <u>A</u> <u>G</u>

^a Amino acids modified by SEC are in bold. Underlined amino acids conform to the animal modification site consensus

Table 2Locations of *O*-GlcNAc modifications on PPV-CP identified using ETD MS

Peptide sequence ^a	Modification site	Observed <i>z</i>	Observed <i>m/z</i>	Calculated <i>m/z</i>
AMADEREDEEEVDAGKPSVVgTAPAAgTSPILQPPPVIQPAPR	Thr19 and Thr24	+5	1,002.6896	1,002.6896
EDEEEVDAGKPSVVTAPAAgTSPILQPPPVIQPAPR	Thr24	+5	795.0067	795.0061
EDEEEVDAGKPSVVgTAPAAATSPILQPPPVIQPAPR	Thr19	+5	795.0065	795.0061
TTASmLNPIFTPAgTTQPATKPVSQVLEHHHHHH	Thr53	+6	675.3354	675.3337
TgTASmLNPIFTPATTQPATKPVSQVLEHHHHHH	Thr41	+6	675.3356	675.3337
TgTASmLNPIFg(TPAT)TQPATKPVSQVLEHHHHHH	Thr41 and (Thr50 or Thr53)	+5	736.1907	736.1890
PVSQVgSGPQLQTFGTyGNEDASPSNSNALVNTNR	Ser65	+5	783.7688	783.7690

^aModified residues are preceded by “g” to signify modification by a single GlcNAc-Gal moiety. Oxidized Met residues are signified by “m” notation