O-Linked N-Acetylglucosamine Proteomics of Postsynaptic Density Preparations Using Lectin Weak Affinity Chromatography and Mass Spectrometry*\S

Keith Vosseller‡§, Jonathan C. Trinidad‡, Robert J. Chalkley‡, Christian G. Specht¶, Agnes Thalhammer¶, Aenoch J. Lynn‡, June O. Snedecor‡, Shenheng Guan‡, Katalin F. Medzihradszky‡, David A. Maltby‡, Ralf Schoepfer¶, Agnes Thalhammer¶, Aenoch J. Lynn‡, June O. Snedecor‡, Shenheng Guan‡, Katalin F. Medzihradszky‡, David A. Maltby‡, Ralf Schoepfer¶, and Alma L. Burlingame‡

O-GlcNAc is a widespread dynamic carbohydrate modification of cytosolic and nuclear proteins with features analogous to phosphorylation. O-GlcNAc acts critically in many cellular processes, including signal transduction, protein degradation, and regulation of gene expression. However, the study of its specific regulatory functions has been limited by difficulties in mapping sites of O-GlcNAc modification. We report methods for direct enrichment and identification of in vivo O-GlcNAc-modified peptides through lectin weak affinity chromatography (LWAC) and mass spectrometry. The effectiveness of this strategy on complex peptide mixtures was demonstrated through enrichment of 145 unique O-GlcNAc-modified peptides from a postsynaptic density preparation. 65 of these O-GlcNAc-modified peptides were sequenced and belonged to proteins with diverse functions in synaptic transmission. β-Elimination/Michael addition, MS\S on O-GlcNAc neutral loss ions, and electron capture dissociation were shown to facilitate analysis of O-GlcNAc-modified peptides/sites from lectin weak affinity chromatography enriched postsynaptic density samples. Bassoon and Piccolo, proteins critical to synapse assembly and vesicle docking, were extensively modified by O-GlcNAc. In some cases, O-GlcNAc was mapped to peptides previously identified as phosphorylated, indicating potential interplay between these modifications. Shared substrate amino acid context was apparent in subsets of O-GlcNAc-modified peptides, including “PVST” and a novel “TTA” motif (two hydroxyl-containing amino acids adjacent to an alanine). The results suggest specific roles for O-GlcNAc modification in synaptic transmission, establish a basis for site-specific regulatory studies, and provide methods that will facilitate O-GlcNAc proteome analysis across a wide variety of cells and tissues. Molecular & Cellular Proteomics 5:923–934, 2006.

Although phosphorylation is a major regulatory mechanism, alternative post-translational modifications such as acetylation, methylation, and O-GlcNAc modification are now understood to play critical roles in cellular processes. O-GlcNAc is an enzyme-mediated cytosolic and nuclear carbohydrate modification of serines and threonines by N-acetylglucosamine which is found in all multicellular eukaryotes on diverse functional classes of proteins (1, 2). O-GlcNAc modification is responsive to a variety of cell stimuli (3, 4) and in some cases is reciprocal with phosphorylation (5), leading to models of functional interplay between these two modifications. O-GlcNAc modification functions in diverse cellular processes such as signal transduction (6), protein degradation (7), control of gene expression (8), and cell cycle progression (9).

There is evidence that O-GlcNAc modification has uniquely important roles in the brain. Enzymes that catalyze addition (O-GlcNAc transferase (OGT)\S) and removal (O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidase) of the modification are most highly expressed in brain (10, 11). Brain-specific knock-out of OGT leads to abnormal neuronal development and motor defects resulting in neonatal death (12). Increased levels of O-GlcNAc modification have been linked to neuronal apoptosis in mouse hippocampus (13). Reciprocal O-GlcNAc/phosphorylation has been observed in cerebellar neurons (14) and appears to regulate Tau participation in neurofibrillary tangles in Alzheimer disease (15). Finally O-
O-GlcNAc modification is abundant at nerve terminals (16, 17) and immunolocalizes to both pre- and postsynaptic sites. A recent study identified O-GlcNAc-modified peptides belonging to 25 unique proteins in whole brain (18), expanding our knowledge of O-GlcNAc modification in this tissue.

Progress in understanding site-specific regulatory functions of O-GlcNAc modification has been limited in part by difficulties associated with mapping modification sites. As is the case with most post-translational modifications, O-GlcNAc often modifies low abundance regulatory proteins substoichiometrically and is therefore represented at low levels in the proteome. O-GlcNAc modification is also labile during CID in mass spectrometry. The energy imparted by CID mainly dissociates GlcNAc from the peptide prior to backbone fragmentation, leaving behind no mass signature and making peptide sequencing and site assignment difficult. These challenges have prompted development of various strategies for enrichment and stabilization of O-GlcNAc modification prior to mass spectrometry (18–24). As all of these methods involve multistep chemical and/or enzymatic derivatization of O-GlcNAc, low sensitivity and lack of specificity are potential concerns (see “Discussion” for further details). Novel fragmentation strategies such as electron capture dissociation (ECD) in Fourier transform ion cyclotron resonance mass spectrometry may preserve modifications that are labile during CID (25). However, the effectiveness of ECD in O-GlcNAc proteome analysis has not been demonstrated and would depend on development of a technique for enrichment of low abundance, native O-GlcNAc-modified peptides from in vivo samples.

Phosphorylation site mapping has benefited from direct phosphopeptide enrichment through techniques such as IMAC (26, 27). There is currently no comparable method for direct enrichment and analysis of natively in vivo O-GlcNAc-modified peptides. Wheat germ agglutinin (WGA) is a lectin with binding specificity for GlcNAc and sialic acid (28). WGA acts as a dimer containing four carbohydrate binding sites (29) and achieves high affinity interactions with complex carbohydrates through binding multiple sugar moieties simultaneously (30). The single N-acetylgalactosamine moiety of O-GlcNAc has a comparably much lower affinity for WGA. The dissociation constant for free GlcNAc binding to WGA has been measured in the 10 millimolar range (31), suggesting that an O-GlcNAc interaction with WGA would be quite weak. For this reason, it is not unexpected that traditional protocols for WGA capture of glycosylated peptide/protein involving binding, washing, and specific elution steps (although effective for complex carbohydrates) have not been effective in isolating O-GlcNAc-modified peptides.

The postsynaptic density is a synaptic structure containing receptors, signaling molecules, and cytoskeletal proteins critical for responding to neurotransmitters. Proteomic analysis of purified postsynaptic density fractions have led to identification of about 500 unique proteins and several hundred phosphorylation sites (32–34). Here we describe novel WGA-based lectin weak affinity chromatography (LWAC) for enrichment of O-GlcNAc-modified peptides coupled to mass spectrometric approaches uniquely suited for O-GlcNAc modification characterization (including ECD). These strategies were applied to analysis of O-GlcNAc-modified peptides from a mouse brain postsynaptic density preparation. The results suggest specific roles for O-GlcNAc modification in synapse assembly, synaptic vesicle movement, and postsynaptic signaling. This work in combination with an accompanying report (Trinidad et al. (55)) on the phosphoproteome of postsynaptic density preparations suggests models of complex protein regulation at the synapse through the interplay of O-GlcNAc modification and phosphorylation.

**MATERIALS AND METHODS**

**LWAC WGA Isocratic HPLC—**WGA coupled to agarose (Vector Laboratories) washed with WGA buffer (25 mM Tris, pH 7.8, 300 mM NaCl, 5 mM CaCl₂, 1 mM MgCl₂) was either packed into an empty 200-mm-long glass Tricorn HPLC column with a 5-mm inner diameter (Amersham Biosciences) or (for use with postsynaptic density sample) was packed into an opaque 12-m (about 39 feet) length of Teflon tubing with outer diameter of 1.59 mm (1/16 of an inch) and inner diameter of 0.1 mm (0.04 inches) (Upchurch Scientific) that was fitted with polyetheretherketone unions at its ends containing 0.5-μm frits to create a column. 5 pmol of a trypptic digest of commercially obtained bovine crystallin protein (Sigma) or 5 pmol of a trypptic digest of bovine α-S2 casein (Sigma) and β-casein (Sigma) spiked with 5 pmol of the O-GlcNAc-modified synthetic peptide PSVPV(S-O-GlcNAc)G-SAPGR derived from the UL32 protein of human cytomegalovirus and synthesized as described previously (35) (a gift from Gerald Hart) was dried, suspended in 40 μl of WGA buffer, and loaded into a 50-μl loop on an AKTA Purifier (Amersham Biosciences) HPLC system. O-GlcNAc-modified peptides were enriched by an isocratic 100% WGA buffer at a flow rate of 0.15 ml/min, and 1-min fractions were collected, acidified with addition of a 0.5% volume of formic acid, and desalted using C₁₈ Zip-Tips for subsequent analysis. For MALDI-TOF analysis, samples were dissolved in α-cyano-4-hydroxycinnamic acid matrix, and 1/5 of the sample was spotted onto a MALDI plate for analysis using a Voyager DE-STR (Applied Biosystems) instrument in reflectron mode. For LC-MS/MS, the sample was dissolved in 0.1% formic acid, and 1/5 of the sample was analyzed. For WGA enrichment of postsynaptic density tryptic digest, 100–600 μg of sample in 40 μl of WGA buffer was loaded into a 50-μl loop on an AKTA Purifier (Amersham Biosciences) HPLC system, and a method triggering injection, 120 min of isocratic 100% WGA buffer flow at 0.15 ml/min, UV monitoring, and fraction collection at 1-min intervals was initiated. Combined fractions of interest were acidified by addition of a 0.5% volume of formic acid and desalted using C₁₈ Zip-Tips for subsequent LC-MS/MS analysis. During WGA isocratic chromatography, the pressure of the HPLC system was observed to rise slowly due to the compression of agarose resin. The pressure was never allowed to exceed 5 millipascals. In cases where multiple sequential runs were performed, the column was inverted after each run to release the compression of the preceding run. For reuse and storage of WGA columns, WGA buffer containing 0.08% sodium azide and 20 mM GlcNAc to stabilize the lectin was occasionally used. In cases where chromatographic effectiveness appeared to decrease, a stringent wash using acetic acid, pH 3, plus 1 M NaCl was used to remove nonspecifically bound material and potentially bound complex glycoconjugates that may interfere with separations.
Postsynaptic Density Fraction Preparation and Tryptic Digestion—

Postsynaptic density fractions were prepared as described previously (34) (see also accompanying paper, Trinidad et al. (55)) except that where noted 20 μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAC) (Carbogen) was included up to the step of synaptosome purification. Because PUGNAC was observed to bind to the WGA column and interfere with chromatography, it was omitted from the final purification steps (i.e., from the second sucrose gradient onward). Pelleted postsynaptic density was suspended in 500 μl of 6 mM guanidine HCl, diluted with 50 mM NH₄HCO₃ to below 1 M guanidine HCl, reduced in 2 mM tris(2-carboxyethyl)phosphine, alkylated with 10 mM iodoacetamide in the dark, and digested with 1/50 to 1/60 by weight modified sequencing grade trypsin (Promega) overnight at 37 °C. Insoluble material was removed from the postsynaptic density digest by centrifugation, and the sample was desalted using C₁₈ filled Sep-Paks (Millipore) and dried in a SpeedVac.

β-Elimination/Michael addition with DTT (BEMAD) Treatment of WGA-enriched Postsynaptic Density Fractions—Dried sample was dissolved in 100 μl of β-elimination/Michael addition solution (pH 12.5–13) containing 1.5% triethyleneamine (Pierce), 0.15% NaOH, and 20 mM DTT (Sigma) and incubated for 1.5 h at 52 °C, acidified to below pH 5 with addition of TFA to 2%, desalted with C₁₈ Zip-Tips, and dissolved in 0.1% formic acid for LC-MS/MS analysis.

Nano-LC-ESI-Qq-TOF Mass Spectrometry Analysis—Peptides were analyzed by LC-MS/MS on either a QSTAR Pulsar mass spectrometer (MD Sciex, Concord, Ontario, Canada), an LTQ, or LTQ-FT (Thermo Finnigan) system operating in positive ion mode. Chromatography was by nanoflow HPLC using the 1100 series HPLC (Agilent) at a flow rate of 300 nl/min. Separation was achieved by a gradient of increasing acetonitrile in water (2–34%) for durations ranging from 45 to 80 min using 0.1% formic acid as the ion pairing agent on a capillary 75-μm-inner diameter column self-packed with Jupiter Proteo C₁₁₂ (Phenomenex) chromatographic support. The LC eluent was directed to microion spray sources. MS and MS/MS data on the QSTAR were recorded with 4-s cycle times as described previously (36). On the LTQ automatic gain control was set at 3 × 10⁴ ions for MS, 1 × 10⁵ for selected ion monitoring scan, and 2 × 10⁵ ions for MS² with triggering of MS³ on MS/MS ions showing neutral loss of either 102 or 68. LTQ-FT data were all acquired in the FT. Automatic gain control was 3 × 10⁴ ions for MS, 5 × 10⁴ ions for selected ion monitoring scans, and 1 × 10⁴ ions for ECD MS. In all cases, singly charged ions were excluded, and dynamic exclusion was used to prevent repetitive selection of the same ions within a preset time. Collision energies were programmed to be adjusted automatically according to the charge state and mass value of the precursor ion. Peak lists for database searching of QSTAR data were created using a script from within the Analyst software that “smoothed” the data by merging data points in the MS spectra within 0.02 Da of each other prior to centroiding, and data points within 0.05 Da of each other in the MS/MS spectra were merged prior to centroiding. Peak lists for LTQ and LTQ-FT data were created using Mascot Distiller version 1.1.2.0 (Matrix Science). This software uses its own peak detection algorithm to create peak lists from profile data. MS/MS data was acquired in centroid mode, so the Distiller converted this into a profile format assuming 20 peaks per dalton for data acquired in the linear trap and 100 peaks per dalton for data acquired in the FT. Peak detection and deisotoping is then performed, and then the data are converted back to a centroided peak list. The peak lists were submitted to an in-house version of the search algorithm Mascot version 1.8 (Matrix Science). Code was appended to Mascot such that the neutral loss of O-GlcNAc (−203.08) from serines and threonines could be searched as a variable modification to peptides. For analysis of BEMAD-labeled peptides, code was appended to allow variable modification search for the stable addition of DTT (+136.1 Da) to serine and threonine or to cysteine (+120.1 Da). The allowed mass tolerance between expected and observed masses for QSTAR data was ±80 ppm for MS and ±0.2 dalton for MS/MS fragment ions; for LTQ data it was ±0.3 dalton for MS and 0.5 dalton for MS/MS. Searches were performed against both the nonredundant National Center for Biotechnology Information database (NCBI, April 12, 2005) containing 2,869,560 entries and Swiss-Prot (April 12, 2005) containing 190,255 entries. Mascot searches were performed on rodent subdatabases and on a customized subdatabase containing proteins identified as components of the postsynaptic density fraction (1350 entries). The Mascot algorithm was set to search for neutral loss fragment ions in predicting O-GlcNAc-modified peptides. Identiﬁcations suggested by automated searching were manually analyzed to assess O-GlcNAc modiﬁcation by the presence of diagnostic ions as described in the text and further analyzed to assess assignment of major ions in MS/MS including potential ions retaining the mass of O-GlcNAc that Mascot would not have predicted. Data from LTQ-FT were manually interpreted. As manual inspection of MS/MS invariably revealed additional sequence information from O-GlcNAc-modified fragments, identiﬁcations were often made with greater confidence than would be indicated by the Mascot conﬁdence score. To detect peptides giving rise to O-GlcNAc-speciﬁc diagnostic ions, a script was written in Perl to search for the combination of m/z 204.08 (tolerance, ±0.01 Da) and the m/z of intact precursors having lost O-GlcNAc (−203.08 Da with tolerance of ±0.1 Da) in centroided QSTAR MS/MS peak lists generated by the Analyst software (This script is publicly available at ms-facility.ucsf.edu/software/). To facilitate determination of speciﬁc O-GlcNAc modiﬁcation sites, a script was written in Perl to search for MS/MS fragment ions retaining O-GlcNAc (This script is publicly available at ms-facility.ucsf.edu/software/). Peptide sequence and elution time information was taken from Mascot and Protein Prospector search engine results, and the hypothetical masses of b and y ions retaining O-GlcNAc were generated and searched for in the centroided peak lists with a tolerance of ±0.2 Da. Any peak above 1% of the intensity of the top three ions was considered.

RESULTS

**LWAC Enrichment of O-GlcNAc Peptides**—We considered that separation of O-GlcNAc-modified peptides from unmodiﬁed peptides might be achieved through their retardation on a WGA afﬁnity column during isocratic HPLC. During such “weak afﬁnity” chromatography, O-GlcNAc-modified peptides migrating slower through a WGA column would be expected to elute later than unmodiﬁed peptides and could thus be enriched. To test this hypothesis, a tryptic digest of α and β-casein containing the synthetic O-GlcNAc-modiﬁed peptide PVPVS-O-GlcNAcGSPAGR was injected onto a 20-cm long WGA column with a 5-mm inner diameter in line with an isocratic HPLC system at a ﬂow rate of 0.15 ml/min, 40 fractions eluting from the WGA column at 1-min intervals were analyzed by MALDI-TOF. The O-GlcNAc-modiﬁed peptide MH⁺ at m/z 1313.3 was observed along with α and β-casein peptides in the starting mixture (Fig. 1A). The O-GlcNAc-modiﬁed peptide was detected as the major component in fraction 29 (Fig. 1B) and was the only ion detected in fraction 33 (Fig. 1C), demonstrating enrichment and recovery in a relatively pure form and indicating the potential usefulness of LWAC. The signiﬁcant overlap in elution of the O-GlcNAc-
modified peptide and unmodified peptides in fraction 29 (Fig. 1B) indicated that further optimization of O-GlcNAc peptide enrichment would be desirable.

\(\alpha\)A-Crystallin contains a well characterized site of O-GlcNAc modification within the tryptic peptide AIP(S-O-GlcNAc)R (37) estimated to occur at relatively low stoichiometry (about 10%). Thus, \(\alpha\)A-crystallin has been used as a model protein for testing O-GlcNAc enrichment and site mapping strategies (18, 20, 38). During LC-MS/MS of an unfractinated \(\alpha\)A-crystallin tryptic digest, \([M + 2H]^2^+\) m/z 423.2 corresponding to the anticipated O-GlcNAc-modified peptide AIP(S-O-GlcNAc)R was observed most intensely at 19.1 min as a minor component in a mixture of co-eluting ions (Fig. 1D). In fraction 33 of LWAC enrichment of the \(\alpha\)-crystallin tryptic digest, the O-GlcNAc-modified peptide \([M + 2H]^2^+\) m/z 423.2 was observed to be the most intense ion at 19.1 min of LC-MS with little apparent co-elution of other ions (Fig. 1E). In the LWAC enriched state, this ion was easily targeted for MS/MS, confirming its identity as AIP(S-O-GlcNAc)R (Supplemental Fig. 1) from \(\alpha\)A-crystallin.

**LWAC Enrichment of O-GlcNAc Peptides from the Postsynaptic Density**—The purity of a postsynaptic density preparation was established by demonstrating enrichment of known postsynaptic density components using Western blotting as described previously (34) (see accompanying paper by Trinidad et al. (55)). In initial LWAC test studies (see Fig. 1), some overlap in elution of O-GlcNAc modified and non-modified peptides was observed. Therefore, in LWAC enrichment of the tryptic postsynaptic density preparation, the WGA column was lengthened to increase the time of lectin interaction with O-GlcNAc-modified peptides and thus enhance separation from non-modified peptides. A column of agarose-coupled WGA packed in a 12-m (about 39 feet) length of Teflon tubing with a 0.1-mm (0.004 inch) inner diameter and a 1.59-mm (\(\frac{1}{16}\) inch) outer diameter was connected in line with the HPLC system. Injection of 100 \(\mu\)g of a postsynaptic density fraction digested with trypsin was followed by isocratic HPLC. A major UV peak was observed to elute between about 70 and 80 min with a distinct much smaller “LWAC enriched” peak of UV elution following at about 81–86 min (Fig. 2A). Fractions corresponding to the LWAC enriched peak were pooled and desalted for analysis by LC-MS/MS on a QSTAR mass spectrometer (Fig. 2B). An example of a precursor at \([M + 2H]^2^+\) m/z 644.9 (Fig. 2C) selected for MS/MS (Fig. 2D) is shown. A prominent ion at m/z 204.08 corresponds to the GlcNAc oxonium ion generated by dissociation of GlcNAc from the peptide. The ion at m/z 138.06 corresponds to a fragment of GlcNAc itself. Two additional fragment ions observed in Fig. 2D at m/z 534.3 and 1085.7 correspond to the intact precursor peptide having lost the O-GlcNAc modification (−203.08 amu) in doubly and singly charged states, respectively. There is sufficient information in Fig. 2D from gas-phase deglycosylated fragment ions to identify the peptide QLLPSTATVR belonging to the protein Bassoon, a known component of the postsynaptic density fraction. Analysis of the MS/MS in Fig. 2D using the search engine Mascot to interrogate the NCBI database allowing for the variable neutral loss modification of serines and threonines by the mass of O-GlcNAc (203.08) returned QLLPSTATVR modified by O-GlcNAc as the top hit with a confidence score of 49. A y7 ion retaining O-GlcNAc was observed at m/z 934.5 (its appearance facilitated by favored fragmentation N-terminal to proline). However, this information does not allow one to discriminate which serine/threonine is modified in the peptide as O-GlcNAc attachment at either the serine or threonine would produce a modified y7.

A script was written to search MS/MS files for the presence of both m/z 204.08 and an intact precursor having lost the mass of O-GlcNAc (−203.08 amu), which is strongly diagnostic for O-GlcNAc-modified peptides (Fig. 2D, see boxed ions). The script, when used to search LC-MS/MS fragment ions of
non-enriched postsynaptic density peptides, indicated that zero of a total of 501 MS/MS spectra corresponded to an O-GlcNAc-modified peptide. The script, when used to search the LWAC enriched LC-MS/MS fragment ions from Fig. 2B, indicated that 103 of a total of 388 spectra represented O-GlcNAc-modified peptides. The results indicated strong enrichment of O-GlcNAc-modified peptides by LWAC and illustrate the necessity for such enrichment when targeting O-GlcNAc modification in samples of this complexity. LWAC enrichment performed on a subsequent postsynaptic density sample prepared in the presence of PUGNAc (39), an inhibitor of O-glycoprotein 2-acetamido-2-deoxy-D-glucopyranosidase, led to the detection of additional O-GlcNAc-modified peptides. From combined analysis, ions corresponding to a total of 145 unique O-GlcNAc-modified peptides were detected. Of these, 90 were detected in both the first and second postsynaptic density preparations, indicating a high degree of consistency in in vivo O-GlcNAc-modified residues in the postsynaptic density fraction.

O-GlcNAc Site Mapping with BEMAD—Fig. 3A shows an example of how the lability of O-GlcNAc in CID makes the modification site assignment difficult. The MS/MS spectrum of an LWAC enriched postsynaptic density peptide \([M + 2H]^2+\) at \(m/z\) 753.4 indicated O-GlcNAc modification and identified VSTGEVMDYSSK from the protein Piccolo. However, due to complete gas-phase deglycosylation, there was no fragment ion information on the site of O-GlcNAc modification. Previously we have described a strategy (BEMAD) for replacement of O-GlcNAc with a covalent modification (DTT) that is stable during CID and facilitates site assignment (22, 36). The LWAC enriched postsynaptic density sample was BEMAD-treated and analyzed by LC-MS/MS using a QSTAR mass spectrometer. The MS/MS of \([M + 2H]^2+\) at \(m/z\) 719.8 in Fig. 3B corresponded to the originally O-GlcNAc-modified VSTGEVMDYSSK that had been converted to the DTT-modified form. The mass difference between the y10 and y11 ions (223.1) corresponded to the mass of serine plus DTT and identified the original O-GlcNAc modification site (V(S-O-GlcNAc)TGEVMDYSSK). One of the drawbacks of \(\beta\)-elimination/Michael addition strategies in general is the potential for nonspecific derivatization as the chemistry may not distinguish between O-phosphate, O-GlcNAc, and in some cases unmodified serines/threonines (40). Here coupling of BEMAD with prior LWAC-specific enrichment/detection of O-GlcNAc-modified peptides greatly increases the specificity of derivatization.

**Ion Trap MS²/MS³ for O-GlcNAc Peptide Identification**—Although sequence determination of natively O-GlcNAc-mod-
Fig. 3. BEMAD mapping of an O-GlcNAc modification site. An LWAC enriched postsynaptic density sample was analyzed by LC-MS/MS directly, and MS/MS of natively O-GlcNAc-modified [M + 2H]^{2+} 844.9 is shown (A) that identified VSTGEVMDYSSK but did not reveal the site of O-GlcNAc modification (no fragment ions retaining O-GlcNAc). Alternatively the same LWAC enriched postsynaptic density sample was treated with BEMAD prior to LC-MS/MS, and an MS/MS spectrum of [M + 2H]^{2+} at m/z 719.8 is shown (B) that corresponds to the peptide identified in A but with O-GlcNAc replaced by DTT (VI-S-DTT)GEVMDYSSK). The mass difference of 223.1 (serine plus DTT) between the y_{10} and y_{11} ions allows for assignment of the original in vivo O-GlcNAc modification site.

Table I contains a list of 65 O-GlcNAc peptides—Table I contains a list of 65 O-GlcNAc peptides identified from the postsynaptic density preparation. The proteins found to be O-GlcNAc-modified play diverse roles in synaptic vesicle movement and postsynaptic active zone assembly (e.g. Bassoon, Piccolo, and Synapsin), postsynaptic signaling (e.g. Shank 2 and synaptic Ras GTPase-activating protein), cytoskeletal dynamics (e.g. Ankyrin G and neurofilaments L and M), and transcription (e.g. HCF1). Bassoon and Piccolo were extensively O-GlcNAc-modified at 28 and nine sites, respectively. Multiple sites of modification were also observed on Ankyrin G, Synapsin, and neurofilament triplet M. 16 O-GlcNAc modification sites were exactly localized (12 by ECD) as indicated in Table I. It has been observed previously that a subset of O-GlcNAc sites appear to occur within a preferred amino acid context involving prolines and valines proximal to multiple serines/threonines. This study strengthens the characterization of the so-called “PVST” motif as a target for in vivo modification of proteins by OGT as 20 of 65 peptides appeared to contain such a motif (highlighted in bold font in Table I). Additionally a novel shared motif, “TTA” (two hydroxyl-containing amino acids adjacent to an alanine), was observed in a subset of O-GlcNAc-modified peptides identified in this study (highlighted in bold font and italicized in Table I). Two unique peptides from the proteins Synapsin and Piccolo contain a “QTTA” motif, whereas an additional five peptides in Table I contain the exact amino acid sequence TTA. If this motif is relaxed to allow for two hydroxy-containing amino acids adjacent to an alanine, 16 O-GlcNAc-modified peptides in Table I are observed to contain the TTA motif. Notably in the nine cases where exact modification site assignment was possible on peptides containing TTA, O-GlcNAc mapped to within this motif. In several cases, peptides bearing two O-GlcNAc modifications were observed (Table I).

O-GlcNAc Peptide Analysis by ECD—LWAC enriched tryptic postsynaptic density peptides were analyzed by LC-MS/MS using ECD fragmentation on an LTQ-FT instrument. An example of ECD analysis of a precursor [M + 3H]^{3+} at m/z 579.9 leading to unambiguous O-GlcNAc site assignment in the peptide HDTSASTQSTPAS(S-O-GlcNAc)R from Spectrin β2 is shown in Fig 4C. Homogeneous peptide fragmentation is apparent based on the relative intensities and continuous series of both unmodified and O-GlcNAc-modified c ions. The mass difference of 290.12 Da between the c_{13} and the c_{14} ions corresponds to the mass of a serine modified by O-GlcNAc and directly localizes the modification site. Although O-GlcNAc neutral loss ions invariably dominate CID spectra, the presence of the c_{14} exclusively in the O-GlcNAc-modified form indicates the stability of O-GlcNAc in ECD experiments, allowing unambiguous site assignment. In this case, c ion formation is favored due to the basic N-terminal histidine.

Summary of Postsynaptic Density Fraction O-GlcNAc Peptides—Table I contains a list of 65 O-GlcNAc peptides identified from the postsynaptic density preparation. The proteins found to be O-GlcNAc-modified play diverse roles in synaptic vesicle movement and postsynaptic active zone assembly (e.g. Bassoon, Piccolo, and Synapsin), postsynaptic signaling (e.g. Shank 2 and synaptic Ras GTPase-activating protein), cytoskeletal dynamics (e.g. Ankyrin G and neurofilaments L and M), and transcription (e.g. HCF1). Bassoon and Piccolo were extensively O-GlcNAc-modified at 28 and nine sites, respectively. Multiple sites of modification were also observed on Ankyrin G, Synapsin, and neurofilament triplet M. 16 O-GlcNAc modification sites were exactly localized (12 by ECD) as indicated in Table I. It has been observed previously that a subset of O-GlcNAc sites appear to occur within a preferred amino acid context involving prolines and valines proximal to multiple serines/threonines. This study strengthens the characterization of the so-called “PVST” motif as a target for in vivo modification of proteins by OGT as 20 of 65 peptides appeared to contain such a motif (highlighted in bold font in Table I). Additionally a novel shared motif, “TTA” (two hydroxyl-containing amino acids adjacent to an alanine), was observed in a subset of O-GlcNAc-modified peptides identified in this study (highlighted in bold font and italicized in Table I). Two unique peptides from the proteins Synapsin and Piccolo contain a “QTTA” motif, whereas an additional five peptides in Table I contain the exact amino acid sequence TTA. If this motif is relaxed to allow for two hydroxy-containing amino acids adjacent to an alanine, 16 O-GlcNAc-modified peptides in Table I are observed to contain the TTA motif. Notably in the nine cases where exact modification site assignment was possible on peptides containing TTA, O-GlcNAc mapped to within this motif. In several cases, peptides bearing two O-GlcNAc modifications were observed (Table I).
GlcNAc modifications simultaneously were observed, such as QLLPSTATVR from Bassoon (MS/MS/MS provided in Supplemental Fig. 2), which contains the TTA motif.

**DISCUSSION**

We report here direct enrichment and identification of in vivo O-GlcNAc-modified peptides from a mouse brain postsynaptic density preparation through LWAC coupled with mass spectrometry. The results provide the largest number of O-GlcNAc-modified peptides identified in a single study to date and indicate specific roles for O-GlcNAc modification in synaptic vesicle cycling and postsynaptic signaling in excitatory transmission.

Enrichment and Site Mapping of O-GlcNAc-modified Peptides—In contrast to previous methods for proteomic analysis of O-GlcNAc modification (18–20, 23, 24), LWAC directly enriches natively modified O-GlcNAc peptides without the need for chemical or enzymatic tagging or derivatization. Such enrichment was essential for targeting O-GlcNAc modifications in postsynaptic density preparations and led to identification of 63 unique O-GlcNAc-modified peptides by mass spectrometry analysis.

Knowledge of specific O-GlcNAc modification sites is essential to pursuing their regulatory functions. β-Elimination/Michael addition approaches such as BEMAD can help localize O-GlcNAc modification sites but may nonspecifically target O-phosphorylation, O-sulfation, or unmodified residues (40). Here we provide an example of how prior enrichment/detection of O-GlcNAc-modified peptides with LWAC greatly increases the specificity of subsequent β-elimination/Michael addition chemistry (e.g. BEMAD) for O-GlcNAc modification site analysis. Additionally the novel ability to directly enrich in vivo O-GlcNAc-modified peptides with LWAC is particularly useful when combined with emerging fragmentation strategies in mass spectrometry that preserve labile post-translational modifications such as ECD (25) or electron transfer dissociation (41). Here ECD is shown for the first time to effectively characterize in vivo site-specific O-GlcNAc modifications through mapping of 12 sites (Table I). Direct enrichment through LWAC is non-destructive as it does not require removal or derivatization of other post-translational modifications and is thus compatible with the simultaneous study of multiple post-translational modifications in the same sample. This may be of interest given the potential relationship between O-GlcNAc modification and phosphorylation (5) and the increasing appreciation of potentially complex interplay between different post-translational modifications on a single polypeptide.

During LWAC enrichment, peptides carrying two O-GlcNAc modifications were observed to elute slightly later than singly modified peptides (although some overlap during elution occurred) (data not shown). This effect is likely due to the in-
### Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession no.</th>
<th>Mascot score</th>
<th>Peptide sequence(s)</th>
<th>MS³</th>
<th>ECD</th>
<th>BEMAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin polymerization-promoting protein</td>
<td>Q7TQD2</td>
<td>36</td>
<td>APVISgGVTK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shank 2</td>
<td>Q80238</td>
<td>64</td>
<td>SPEVMSTgVSgTgVR (two O-GlcNAc sites)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Synaptotodin</td>
<td>Q8CC35</td>
<td>74</td>
<td>VSAPPSgAASgTFSgR</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrin β2</td>
<td>Q5SQL8</td>
<td>53</td>
<td>HDTSTAQITgSPASSgR</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Catenin-ζ2</td>
<td>O35927</td>
<td>58</td>
<td>TgSgAPgSgSgPgLDSgPSgPVPLQR</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Neurofilament triplet L</td>
<td>P08551</td>
<td>94</td>
<td>SASYSYSAPgVSSgSSgLSgVR</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Synaptic Ras-GTPase-activating protein 1</td>
<td>Q9QHUH6 (rat)</td>
<td>40</td>
<td>QHSSgqTgSSgTgLSSPgLSSgMPASgSSgER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMSY protein</td>
<td>Q8BMB0</td>
<td>25</td>
<td>AVVPVSgK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle tail</td>
<td>Q75UV8</td>
<td>34</td>
<td>LSSLpVSgR</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Myocardin-related transcription factor B</td>
<td>P59759</td>
<td>49</td>
<td>SpTgAPgPMpTNTVSSAK</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Ponsin</td>
<td>O62417</td>
<td>77</td>
<td>TPVDYIDLPSSgSgSPSR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurofilament triplet M</td>
<td>gi</td>
<td>128150</td>
<td>25</td>
<td>GSgSgTgVSgSgSgYKR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internexin</td>
<td>gi</td>
<td>34328368</td>
<td>26</td>
<td>ILSSgSgTgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Host cell factor C1 (HCF1)</td>
<td>P51611</td>
<td>52</td>
<td>SgPITgSTgK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ankyrin G</td>
<td>gi</td>
<td>3885972 (rat)</td>
<td>35</td>
<td>SgAADVISTAK</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Synapsin 1</td>
<td>O88935</td>
<td>67</td>
<td>TPSTAAPQQASAQATgPTRQGQQR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piccolo</td>
<td>Q9QX7</td>
<td>68</td>
<td>SCTAQQPgTgLPEDR</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bassoon</td>
<td>O88737</td>
<td>35</td>
<td>IAVATgEGQQPR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

O-GlcNAc Proteomics of Postsynaptic Density Preparations

A “g” following a serine or threonine denotes specific localization of O-GlcNAc to that site (when underlined) or localization of O-GlcNAc to one of several possible sites if not underlined. Amino acids in bold font correspond to the shared motifs PVST or TTA (in italics). All peptides are singly O-GlcNAc-modified unless otherwise noted in parentheses after the sequence. Exact localization of modification site by ECD and/or BEMAD or enhancement of peptide sequencing by MS³ is noted by “X.”
Increased affinity that peptides bearing two GlcNAc residues would be expected to have for WGA. Complex carbohydrates with terminal GlcNAc or sialic acid may be enriched by WGA. In LWAC of the postsynaptic density preparation, we observed a subset of peptides modified by complex carbohydrate eluting in a significantly later fraction not overlapping with O-GlcNAc-modified peptides (likely due to their relatively stronger affinity for WGA) (data not shown). Thus, LWAC allows for differential enrichment of peptides modified by either O-GlcNAc or complex carbohydrates containing terminal GlcNAc. Succinylation of WGA increases specificity toward GlcNAc over sialic acid (42). However, our preliminary results (data not shown) suggest that some degree of WGA affinity for GlcNAc is compromised by succinylation, making O-GlcNAc-modified peptide enrichment more difficult.

O-GlcNAc Modification of Proteins from Mouse Postsynaptic Density Preparations—Distinct classes of O-GlcNAc-modified proteins were observed in this study. Several presynaptic proteins involved in synaptic vesicle cycling were found to be O-GlcNAc-modified. Bassoon, which is associated with the cytomatrix at active zones mediating vesicle docking and fusion (43), was identified previously as an O-GlcNAc-modified protein through identification of a single O-GlcNAc-modified peptide (18). Here 28 unique O-GlcNAc modification sites were detected on Bassoon. Piccolo, which is structurally homologous with Bassoon (44), was identified previously in an anti-O-GlcNAc immunoprecipitation from mouse brain (22). Here nine Piccolo O-GlcNAc-modified peptides provide direct evidence of this modification. Synapsin represents one of the most intensively site-mapped O-GlcNAc-modified proteins (16). Here two of six previously mapped O-GlcNAc modification sites on Synapsin were observed. It is common to observe certain presynaptic proteins such as Bassoon, Piccolo, and Synapsin in postsynaptic density preparations (discussed in the accompanying paper, Trinidad et al. (55)).

Several synaptic cytoskeletal O-GlcNAc-modified proteins were identified in this study. O-GlcNAc modification of the brain-specific Ankyrin G has been reported previously (45) and localized to a “serine/threonine-rich” domain using an in vitro glycosylation system (45). Interestingly the six Ankyrin G in vivo O-GlcNAc-modified peptides identified in this study cluster in the middle of the serine/threonine-rich domain, a region implicated in axonal membrane targeting (46). A known O-GlcNAc-modified peptide from brain-specific Spectrin β2 (18), which links the cytoskeleton to synaptic vesicle release (47), was also identified here, and the exact site of O-GlcNAc modification on the peptide HDTASQSTTPAS(S-O-GlcNAc)R was determined in ECD MS analysis. Synaptopodin, linked with formation of dendritic spine apparatus and long term potentiation (48), was reported previously as O-GlcNAc-modified (18). Here a novel site of O-GlcNAc modification on synaptopodin is reported. Some of the earliest sites of O-GlcNAc modification were determined on the proteins neurofilaments M and L (49). Mapping of three of four original neurofilament O-GlcNAc modification sites is reproduced in this proteomic analysis.

Several novel O-GlcNAc-modified proteins involved in postsynaptic signal transduction were identified here. Shank 2 is a scaffolding molecule implicated in N-methyl-D-aspartate receptor-mediated signaling and plasticity (50). The small GTPase-activating protein SynGap 1 has been shown to influence synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking (51), and catenin-δ has been linked to growth factor-induced neuronal morphogenesis (52). The chromatin-associated factor HCF was reported previously to be O-GlcNAc-modified and to interact with OGT in the context of Sin3 histone deacetylase-mediated transcriptional repression (53). It will be of interest to examine whether the HCF site-specific O-GlcNAc modification identified here is involved in the link between OGT and Sin3 in transcriptional repression (8).

Substrate Selectivity in O-GlcNAc Modification Events—The overlap in O-GlcNAc-modified peptide identifications across different studies and between independent postsynaptic density preparations indicates that in vivo OGT substrate selectivity is tightly regulated and specific in brain. 14 of
The O-GlcNAc-modified proteins identified in this study are represented by a single O-GlcNAc-modified peptide (Table I). Eight of these 14 peptides contain a PVST motif, which is recognized as a substrate motif for a subset of O-GlcNAc modification sites. O-GlcNAc-modified peptides from extensively modified proteins in this study contained the PVST motif at a much reduced frequency (e.g., three of 26 peptides for Bassoon). This difference may be informative about the regulation of OGT activity. Possibly extensive modification reflects a situation in which OGT is held in physical proximity to proteins like Bassoon and Piccolo in a regulatory complex and would thus require less stringent substrate motif requirements.

Distinct from PVST, the TTA motif was recognized to occur in 16 O-GlcNAc-modified peptides from this study. Where exact modification site assignment was possible, O-GlcNAc attachment occurred within this motif without exception. Determining the extent to which this motif helps direct O-GlcNAc modification will be of interest in future studies. Although a single OGT has been identified in genomes of higher eukaryotes, evidence of distinct substrate motifs (PVST versus TTA) raises the possibility that an unidentified second O-GlcNAc transferase activity may exist.

Relationship between O-GlcNAc and O-Phosphorylation—Regulation of protein function by O-GlcNAc may in some cases involve competition with phosphorylation at specific sites (5). Given the potential relationship between O-GlcNAc modification and phosphorylation, it is of interest to examine overlap in site occupancy by these two modifications on postsynaptic density proteins. The phosphoproteome of postsynaptic density preparations has been examined (accompanying paper, Trinidad et al. (55)) (32, 34). Seven Bassoon O-GlcNAc-modified peptides identified in this study have also been identified as phosphorylated peptides (Table II). Additionally a tryptic peptide from catenin-δ (TSTAPSSPGDVSPLQR) was observed to be both O-GlcNAc-modified and O-phosphorylated (Table II) (accompanying paper by Trinidad et al. (55)). These observations indicate specific examples in which potential functional competition/reciprocity between O-GlcNAc modification and O-phosphorylation may exist.

The case of Bassoon illustrates that primary amino acid sequence analysis alone may miss much of the in vivo character of a molecule as Bassoon is structurally diversified by numerous post-translational modifications. Simultaneous characterization of multiple post-translational modifications on a single protein such as Bassoon is a challenge to proteomics but will be necessary to understand the repertoire of intact modified forms that exist and the likely complex functional interplay of various modification events on this single protein (54). O-GlcNAc is clearly a molecular component at neuronal synapses on a variety of proteins, and it will now be interesting to pursue the specific regulatory roles this modification may play in this specialized domain for neurotransmission.

Acknowledgments—We thank Peter Baker for assistance with data processing. We thank Gerald Hart for the kind gift of the synthetic O-GlcNAc peptide used in this study, and we thank Kirk Hansen for advice on HPLC.

* This work was funded by the Wellcome Trust (to R. S.) and by National Institutes of Health National Center for Research Resources Biomedical Research Technology Program Grants RR01614, RR019934, RR012961, and RR14806 (to A. L. B.). Support of Protein Prospector was from the Vincent J. Coates Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biology, Drexel University College of Medicine, 245 N. 15th St., M. S. 497, Philadelphia, PA 19102. Tel.: 215-762-8789; E-mail: kav27@drexel.edu.

To whom correspondence may be addressed: Dept. of Pharmaceutical Chemistry, University of California, 521 Parnassus Ave., Rm. C18, San Francisco, CA 94143. Tel.: 415-476-5641; E-mail: alb@cgl.ucsf.edu.

REFERENCES


