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COMMUNICATION

Synthesis and protein binding studies of a peptide fragment of clathrin assembly protein AP180 bearing an *O*-linked β-*N*-acetylglucosaminyl-6-phosphate modification†

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A novel post-translational modification of threonine, β-*N*-acetylglucosaminyl-phosphate, was recently discovered on assembly protein AP180, a protein which plays a crucial role in clathrin coated vesicle formation in synaptic vesicle endocytosis (SVE). Herein, we report studies aimed at probing the effect of this modification on binding to proteins in rat brain lysate using pull down experiments with peptide fragments of AP180.

Introduction

Glycosylation and phosphorylation are ubiquitous post-translational modifications of proteins, known to regulate a host of important biological processes.[1] Protein glycosylation occurs primarily on the side chains of asparagine or serine/threonine (Ser/Thr) residues and leads to the formation of *N*-linked and *O*-linked glycoproteins, respectively.[2] β-*O*-linked glycosylation of proteins with a single *N*-acetylglucosamine (*O*-GlcNAc) monosaccharide is a common modification of nuclear and cytoplasmic proteins.[3] Unlike oligosaccharide modifications of proteins, which implicate a host of enzymes for transfer and removal of glycans, addition of the GlcNAc modification to proteins is achieved by a single enzyme, *O*-GlcNAc transferase (OGT) and is removed by a single enzyme, *O*-GlcNAcase.[3a] For most *O*-GlcNAc modified proteins, it is not clear how the turnover of GlcNAc is regulated. The *O*-GlcNAc modification, in a similar manner to phosphorylation, has been implicated in dynamic intracellular signalling roles and, to date, the modification has been shown to play a role in protein interactions, localisation and function.[4]

O-GlcNAc modified proteins are also commonly phosphorylated on Ser and Thr residues. Indeed, a number of well-studied proteins have been shown to have a complex interplay between *O*-GlcNAcylation and phosphorylation.[4,5] Recently, the potential complexity of this interplay was enhanced with the discovery of *O*-GlcNAc-phosphate on Thr-310 of rat assembly protein AP180 (UniProtKB Q05140), a 915 amino acid residue protein enriched in brain nerve terminals that has very high sequence homology in mammals.[6] In this study, AP180 was detected unmodified, *O*-GlcNAc modified or *O*-GlcNAc-phosphate modified at Thr-310, thus opening the possibility for three levels of regulation at this site.[6] Currently, it is not known if this hitherto unknown modification arises from the transfer of GlcNAc-phosphate to Thr-310 or from the phosphorylation of *O*-GlcNAc already present on Thr-310. In addition, the enzymes responsible for the modification have not yet been identified.

AP180 is involved in synaptic vesicle endocytosis (SVE) and has an established role in the assembly of clathrin coated vesicles of uniform size.[7] AP180 is known to bind to the AP-2 complex *via* the alpha and beta subunits and to clathrin heavy chain at an early stage of SVE.[8] SVE is responsible for recycling of the cell membrane with various transmembrane cargo proteins into new synaptic vesicles which can be refilled with neurotransmitter for future rounds of synaptic transmission.[9] AP180 has also been demonstrated to bind weakly to the exocytosis protein vesicle-associated membrane protein 2 (Vamp2 or synaptobrevin 2), a protein which has a role in fusing neurotransmitter filled synaptic vesicles to the membrane during exocytosis.[10] This suggests that AP180 might have a secondary role in sorting Vamp2 into vesicles during SVE. This sorting role would ensure that synaptic vesicles have the correct protein components to achieve efficient exocytosis. AP180 is rapidly dephosphorylated by K^+ stimulation of synaptosomes (*i.e.* when sheared off nerve terminals are depolarised).[11] This implies that the function of AP180 in SVE is phospho-regulated. However, the exact role of AP180 post-translational modifications, in particular the *O*-GlcNAc-phosphate modification, is unknown. In order to probe the importance of the *O*-GlcNAc-phosphate modification, we synthesised an 11-amino acid peptide fragment of AP180 (Fig. 1) centering on Thr-310. Since the exact location of phosphorylation on GlcNAc has not yet been elucidated (*i.e.* on the 2, 3 or 6 positions of the glycan) we surmised that the 6-position was the most likely site to be phosphorylated based on the fact that the mass spectral fragmentation pattern of the carbohydrate moiety of isolated

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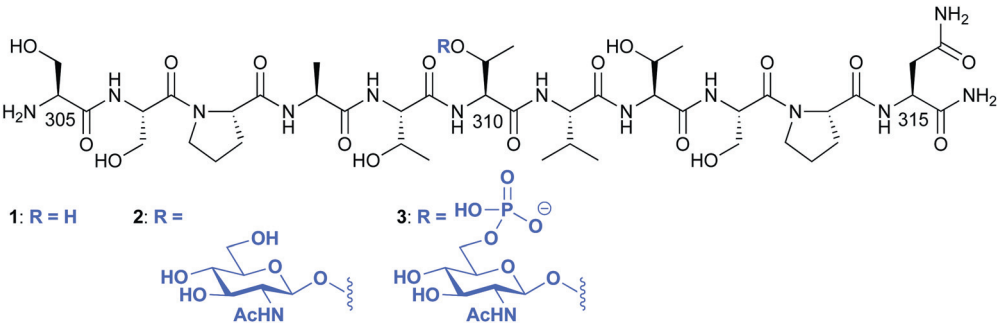
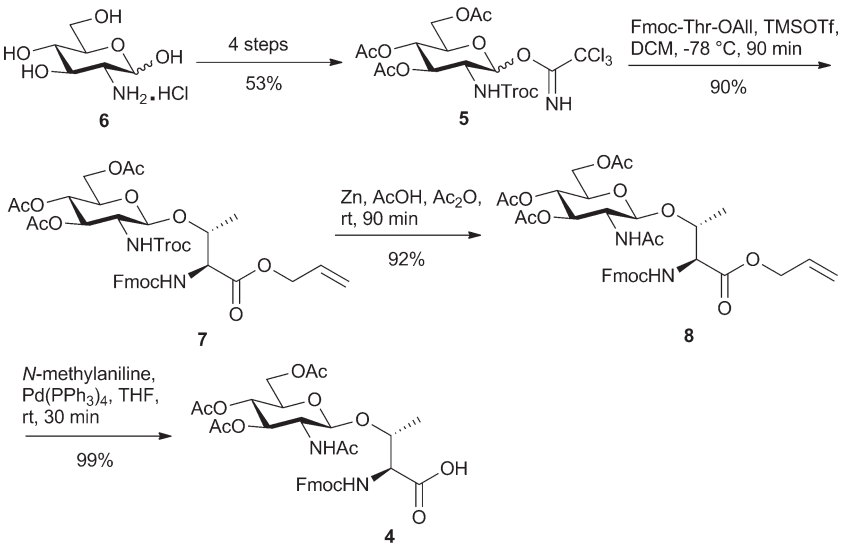


Fig. 1 Target AP180 (305–315) peptides **1–3**.



Scheme 1 Synthesis of Fmoc-β-*N*-acetylglucosaminyl-Thr building block **4**.

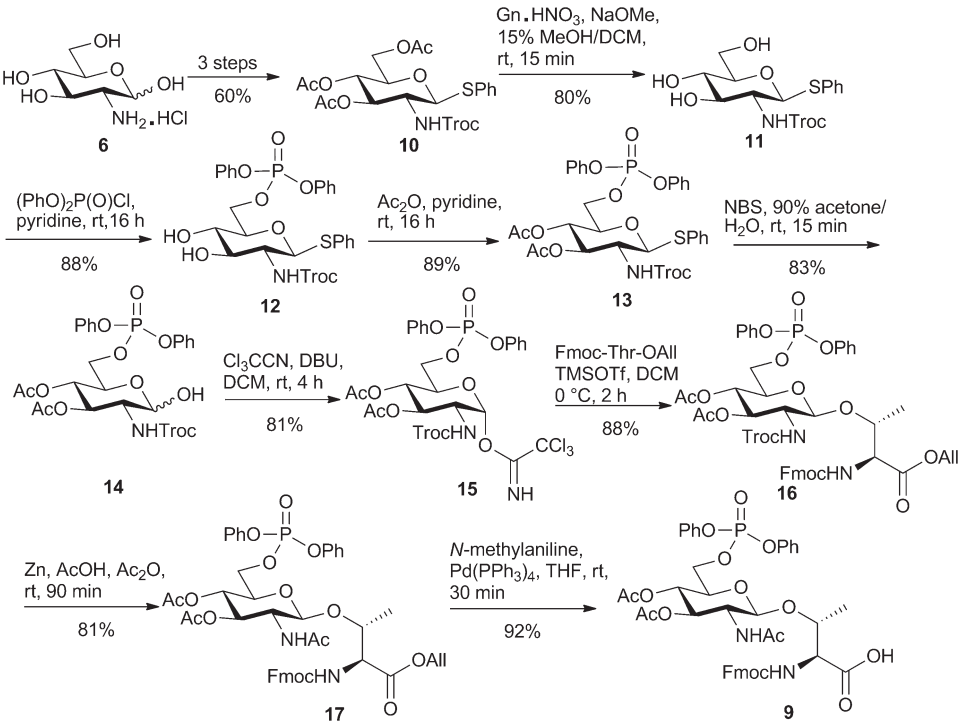
AP180 and synthetic GlcNAc-6-phosphate are almost identical (see ESI†). Two additional precedents lend further support for the regiochemistry of phosphorylation: (1) free GlcNAc is phosphorylated at the 6-position for utilisation in the hexosamine biosynthetic pathway[12] and (2) GlcNAc-6-phosphate was found as a component of complex carbohydrates in bovine colostrums.[13] A further assumption was that the *O*-GlcNAc-phosphate on Thr-310 was in the same β-anomeric configuration as the *O*-GlcNAc modification found on Thr-310.[6]

Three peptides were targeted in this study; AP180 (305–315) possessing no modification on Thr-310 (**1**), AP180 (305–315) bearing an *O*-linked β-GlcNAc modification at Thr-310 (**2**) and AP180 (305–315) containing an *O*-linked β-GlcNAc-6-phosphate modification at Thr-310 (**3**). Once assembled, we envisaged that these peptides could be immobilised onto beads to probe for protein binding partners in synaptosome lysate preparations. This would enable an investigation into the effect of the modifications on AP180-derived peptides on the affinity towards synaptic proteins.

Results and discussion

In order to prepare the AP180-derived peptides **2** and **3** we first needed to synthesise two suitably protected glycosylamino acid building blocks. Synthesis of fluorenylmethoxycarbonyl (Fmoc)-β-*N*-acetylglucosaminyl-Thr **4** began from trichloroacetimidate **5** which was synthesised in four steps and 53% overall yield from D-glucosamine hydrochloride **6** *via* existing literature procedures (Scheme 1).[14] Reaction of **5** with Fmoc-Thr-OAll[15] at −78 °C using TMSOTf as the Lewis acid promoter provided β-glycoside **7** in 90% yield. Reductive cleavage of the trichloroethyl carbamate (Troc) moiety and acetylation of the resulting amine was achieved by treatment with Zn, acetic acid and acetic anhydride, providing **8** in 92% yield. Finally, removal of the allyl ester from **8** using $Pd(PPh_3)_4$ and *N*-methylaniline in THF provided the desired glycosylamino acid building block **4** in excellent yield ready for direct incorporation into Fmoc-strategy solid-phase peptide synthesis (SPPS) of glycopeptide **2**.

Synthesis of Fmoc-β-*N*-acetylglucosaminyl-6-phosphate-derived threonine building block **9** began from thioglycoside **10** which was prepared from D-glucosamine hydrochloride in three steps using modified literature procedures[16] (see ESI†). At this stage treatment of **10** with sodium methoxide in methanol and dichloromethane in the presence of guanidine nitrate enabled selective deprotection of the *O*-acetate groups to afford **11** in 80% yield (Scheme 2). Notably, these conditions prevented concomitant conversion of the Troc-carbamate to a methyl carbamate which occurs under traditional Zemplén conditions.[17] From here, phosphorylation at C6 by treatment with diphenyl-



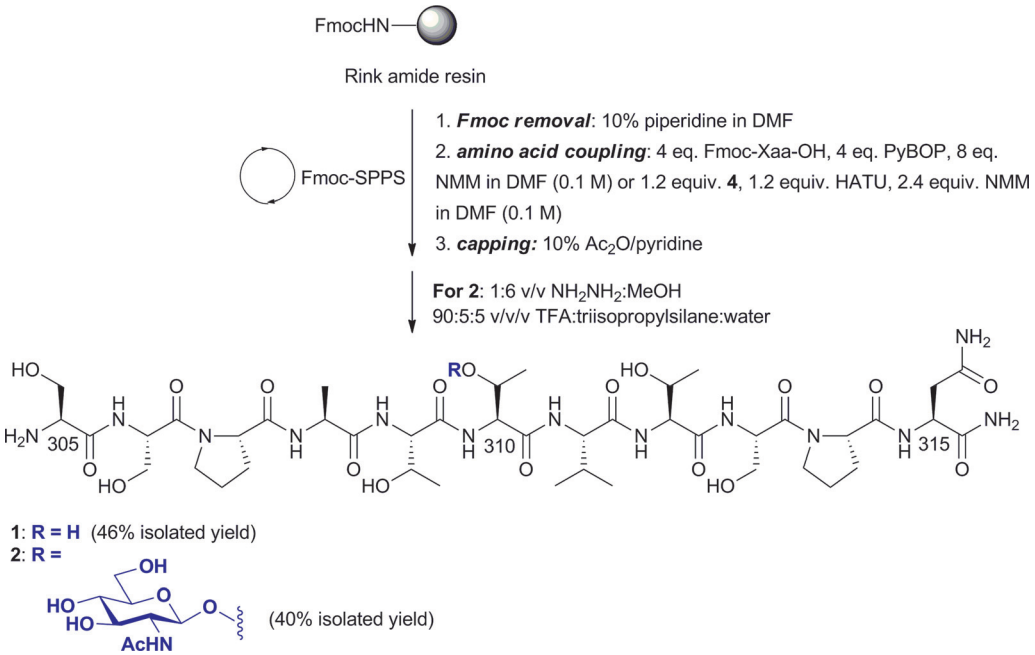
Scheme 2 Synthesis of Fmoc-β-*N*-acetylglucosaminyl-6-phosphoryl-Thr building block **9**.

chlorophosphate and pyridine provided phosphonate **12** in 88% yield. Acetylation of the C3 and C4 alcohols by treatment with acetic anhydride and pyridine provided **13** which was subsequently treated with *N*-bromosuccinimide (NBS) in a mixture of acetone and water to afford hemiacetal **14** in 83% yield. Treatment of **14** with trichloroacetonitrile using 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) as the base provided solely the α-trichloroacetimidate **15** in 81% yield. Reaction of **15** with Fmoc-Thr-OAll at 0 °C using TMSOTf as the Lewis acid promoter provided the desired β-glycosylamino acid **16** in excellent yield (88%). Reductive cleavage of the Troc-carbamate followed by acetylation was achieved by treatment of **16** with zinc dust, acetic acid and acetic anhydride to provide **17** in 81% yield. Finally, Pd(0)-catalysed removal of the allyl ester afforded Fmoc-β-*N*-acetylglucosaminyl-6-phosphoryl-Thr **9** which could be directly incorporated into the Fmoc-strategy SPPS of glycosylphosphopeptide **3**.

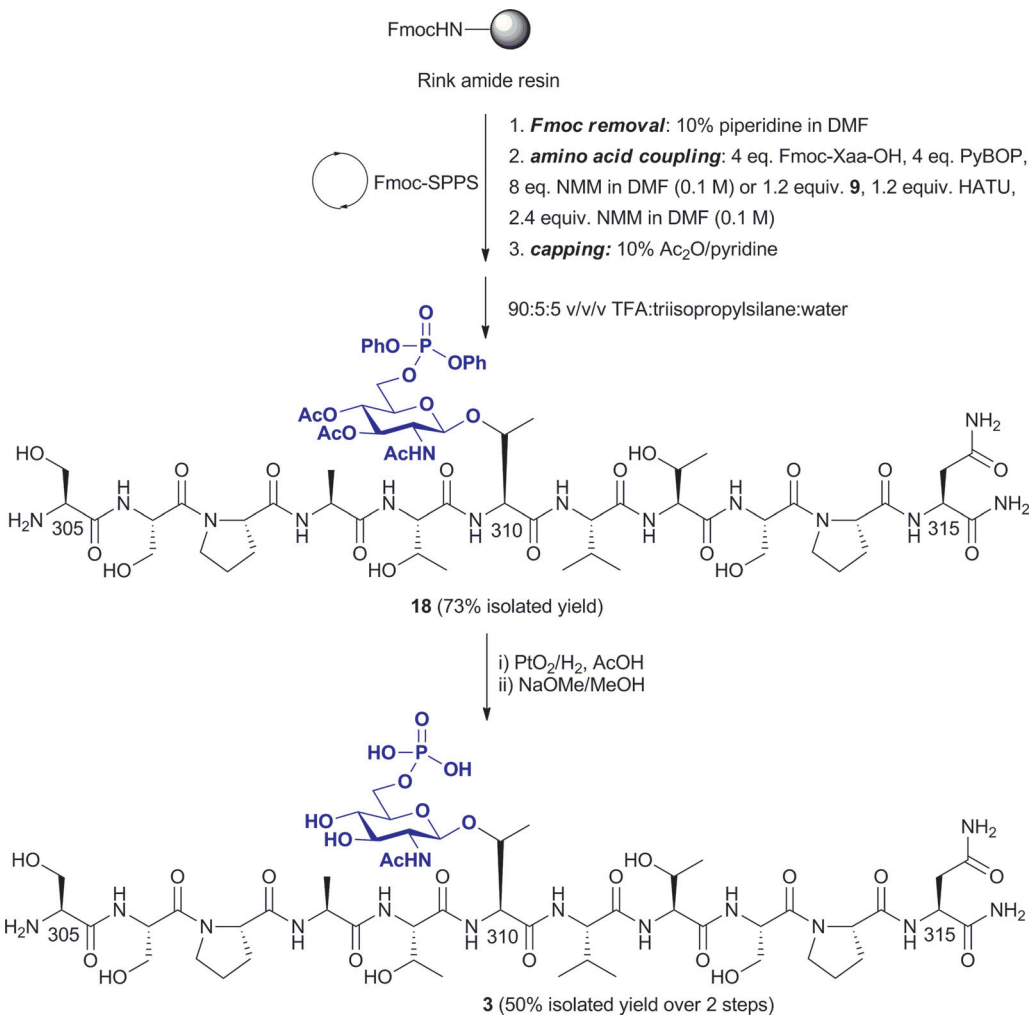
With glycosylamino acid building blocks **4** and **9** in hand, we next embarked on the synthesis of the target peptides. Synthesis of unmodified peptide **1** was achieved *via* standard Fmoc-strategy SPPS starting from Rink amide resin (Scheme 3). Briefly, Fmoc deprotection was performed by treatment with 10% piperidine in DMF, while standard Fmoc-protected amino acids (4 equiv) were coupled using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 4 equiv) as the coupling reagent and *N*-methylmorpholine (NMM, 8 equiv) as the base in dimethylformamide (DMF) for 1 hour. After each coupling step, any unreacted resin-bound amines were acetylated using a capping solution (comprising 10% acetic anhydride in pyridine). After the sequence had been fully assembled, the peptide was cleaved from the resin using an acidic cocktail and purified by reversed-phase HPLC to afford **1** in 46% isolated yield (based on the resin loading of the first amino acid). The synthesis of glycopeptide **2** was carried out using the identical Fmoc-strategy SPPS protocol as that described for **1**, however, to prevent excess expenditure of glycosylamino acid **4**, coupling was carried out using a slight excess (1.2 equiv) of **4**, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent and NMM as the base and the reaction time extended to 16 h. After the glycopeptide had been fully assembled on the resin, the *O*-acetate protecting groups on the β-GlcNAc moiety were removed *via* hydrazinolysis. Acidic cleavage from the resin followed by reversed-phase HPLC purification then provided **2** in 40% isolated yield (based on the resin loading of the first amino acid).

Synthesis of AP180 (305–315) glycosylphosphopeptide **3** bearing the β-*N*-acetylglucosaminyl-6-phosphate modification at Thr-310 was also synthesised *via* Fmoc-strategy SPPS using building block **9** (Scheme 4). Cleavage of the peptide from the resin followed by purification by reversed-phase HPLC provided protected glycosylphosphopeptide **18** in 73% yield (based on the resin loading of the first amino acid). Hydrogenolysis of the two phenyl ester phosphate protecting groups using platinum oxide and H_2 in acetic acid followed by deacetylation under Zemplén conditions provided **3** in 50% isolated yield over the two steps after purification by reversed-phase HPLC.

Having successfully prepared peptide **1**, glycopeptide **2** and glycosylphosphopeptide **3**, these peptides were next immobilised onto *N*-hydroxysuccinimide-derived sepharose *via* their N-termini. To compare protein binding partners, the beads with peptides **1**, **2** and **3** attached, or the beads alone, were separately exposed to rat brain synaptosome lysate preparations in pull down experiments. After washing the beads, the bound protein was digested with trypsin and analysed by LC-MS/MS. The



Scheme 3 Preparation of AP180 (305–315) peptide **1** and glycopeptide **2** *via* Fmoc-strategy SPPS.



Scheme 4 Preparation of AP180 (305–315) glycosylphosphopeptide **3** *via* Fmoc-strategy SPPS.

sequence information of the resulting peptides was compared to theoretical sequences in a protein database. Confidently matched tryptic peptides were used to identify proteins that were bound to the beads. Poor matches were discarded. Peptides that were not confidently identified at least three times were discarded to avoid false positive identifications (see details in ESI†). A minimum of two peptides unique to each protein were used for quantification (see ESI, Table S1,† for the list of identified peptides). In total, 293 proteins met the minimum criteria and were examined for differential binding to peptides **1**, **2** or **3** bearing the different modifications (see ESI, Table S2,† for the list of identified proteins). A subset of 104 proteins showed significant differential binding to **1**, **2** or **3** compared to beads alone or another peptide ($p < 0.05$, *t*-test). A subset of 19 proteins had significantly different binding upon comparison of the unmodified peptide **1** to *O*-GlcNAc peptide, **2**, or upon comparison of **2** to the *O*-GlcNAc-6-phosphate-derived peptide **3** (Fig. 2). This final protein group, determined after discarding five proteins that were primarily localised to mitochondria, became the focus of this study.

Clathrin heavy chain and the AP-2 complex alpha and beta subunits were detected, but did not exhibit any significantly different binding to the 11-amino acid peptides **1–3** (see ESI, Table S2†). Likewise, there was no significant difference in the binding of Vamp2. Since Vamp2 is small (116 amino acids) and has high homology with other Vamp family members (rat Vamp1 is 77% identical to Vamp2 using EMBOSS needle sequence alignment) there are very few unique tryptic peptides which can be utilised for quantification. Therefore, we combined Vamp1 and Vamp2 peptides for quantification. The combined Vamp1/2 data revealed a 2-fold preference for Vamp1/2 binding to peptide **2** with an *O*-GlcNAc modification compared to non-modified peptide **1** ($p = 0.039$). A 2-fold relative decrease in binding was observed for *O*-GlcNAc-6-phosphate containing peptide **3**, however, this was not significant ($p = 0.059$). Interestingly, three other exocytic proteins involved in vesicle fusion also bound the AP180 peptides and showed a similar binding preference pattern. In particular, Ras-related protein Rab3A and Syntaxin 1a (Stx1a) bound to *O*-GlcNAc-modified **2** in preference to unmodified **1** (2.6-fold difference, $p = 0.019$ and 2.5-fold difference, $p = 0.00007$, respectively), similar to Vamp1/2. Moreover, they bound **2** in preference to **3**, bearing the *O*-GlcNAc-6-phosphate modification (1.9-fold difference, $p = 0.035$ and 2.5-fold difference, $p = 0.036$, respectively). The third exocytic protein synaptosomal-associated protein 25 (Snap25) bound unmodified peptide **1** in preference to *O*-GlcNAcylated peptide **2** (2-fold difference, $p = 0.0014$).

Taken together, these results suggest that *O*-GlcNAc can serve as a binding motif for proteins in brain lysate (*e.g.* for Vamp1/2, Rab3A and Stx1a) and that addition of a phosphate moiety at the 6-position of the glycan was capable of altering *O*-GlcNAc binding (Rab3A and Stx1a). In addition, modification of the hydroxyl side chain in Thr-310 can hinder binding to certain proteins (*e.g.* Snap25). As such, it is possible that these modifications are responsible for regulating binding *in vivo*. Should these results be confirmed, phosphorylated *O*-GlcNAc on AP180 could serve a role in regulating the localisation of exocytic fusion proteins at the membrane or sorting transmembrane cargo (*e.g.* Vamp1/2) into synaptic vesicles. Although these studies have only been conducted on modified peptides, the differential binding of the AP180-derived peptides to Vamp1/2 suggested from these preliminary studies is intriguing because the AP180 family member, clathrin assembly and lymphoid myeloid leukemia (CALM) protein, binds strongly to Vamp2 *via* its N-terminal domain[10,18] and has a role in regulating the localisation of Vamp2. Since the CALM N-terminal domain is nearly the same as AP180 (81% identical, 93% similar, at amino acids 1–289 using EMBOSS needle sequence alignment) there may be an unidentified requirement of additional protein folding or modification to allow similar strong binding to AP180.

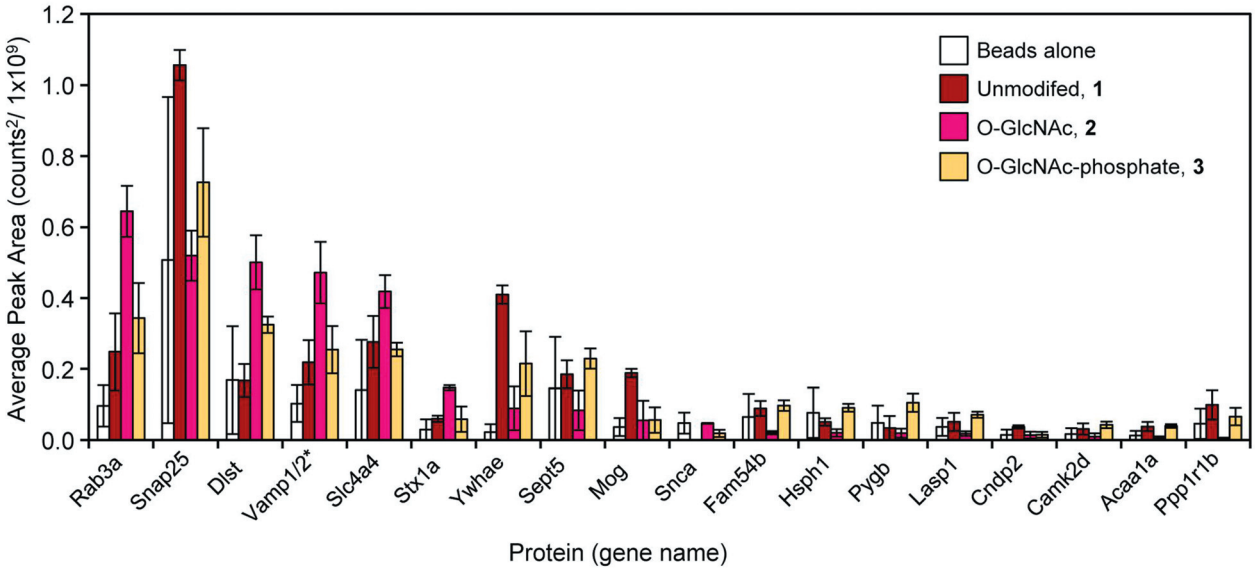
Apart from metabolic proteins (Dlst, Pygb and Acaa1a) there was a regulator of cytoskeleton protein (Lasp1), a filamentous protein (Sept5), a component of the myelin sheath (Mog), a dipeptidase (Cndp2), a protein of unknown function (Fam54b), an anion transport protein (Slc4a4), a chaperone protein (Hsph1) and a Parkinson's disease related protein (Snca).[19] The Heat shock protein 105 kDa (Hsph1) was an example where binding to the *O*-GlcNAc-6-phosphate-modified peptide, **3**, was preferred over binding to the *O*-GlcNAc peptide, **2** (4.5-fold difference, $p = 0.005$). Hsph1 is responsible for inhibition of the ATPase and chaperone activity of Hsc70,[20] a protein involved in uncoating newly formed clathrin coated synaptic vesicles.[21] A protein kinase subunit (Camk2d), and a protein phosphatase subunit (Ppp1r1b) also preferred to bind to peptide **3** over unphosphorylated *O*-GlcNAcylated peptide **2** (4.5-fold difference, $p = 0.032$). Unexpectedly, the phospho Ser/Thr binding 14-3-3 protein epsilon (Ywhae) preferred the unmodified peptide **1**.

This study represents an important first step in elucidating potential protein binders of AP180 in rat brain lysate. However, given that these binding studies were only conducted on 11 amino acid fragments of AP180, more detailed studies on the full length protein are now required to ascertain if any of these novel binding proteins are genuine AP180 binding partners *in vivo*. However, these results clearly demonstrate the potential for *O*-GlcNAc and *O*-GlcNAc-6-phosphate to alter peptide–protein interactions.

Conclusions

In summary, we have successfully developed a high yielding chemical synthesis of glycosylamino acid building block **9** which possesses the β-*N*-acetylglucosaminyl-6-phosphate post-translational modification recently discovered at Thr-310 of AP180. Three 11-amino acid peptides corresponding to AP180 (305–315) bearing no modification at Thr-310 (**1**), β-*O*-GlcNAc at Thr-310 (**2**) and β-*O*-GlcNAc-6-phosphate at Thr-310 (**3**) were subsequently synthesised and attached to a sepharose solid support for pull down assays with rat brain synaptosome lysate. Importantly, the differentially modified peptides were shown to have varied binding affinities for numerous proteins in the lysate, which may suggest the possibility of multi-level regulation *in vivo*. The β-*O*-GlcNAc-6-phosphate modification on the AP180 fragments did not appear to alter binding to well known protein partners, clathrin and the AP-2 complex, but showed differential binding to a recently identified AP180 binding partner, Vamp2 and other exocytic proteins.

A)



B)

Gene Name	Accession	Protein Name	Molecular Function
Rab3a	P63012	Ras-related protein Rab-3A	catalytic activity; enzyme regulator activity; metal ion binding; nucleotide binding; protein binding
Snap25	P60881	Synaptosomal-associated protein 25	protein binding; transporter activity
Dlst	Q01205	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	catalytic activity; protein binding
Vamp1/2	Q63666/ P63045	Vesicle-associated membrane protein 1/2	protein binding
Slc4a4	Q9JI66	Electrogenic sodium bicarbonate cotransporter 1	transporter activity
Stx1a	P32851	Syntaxin-1A	protein binding
Ywhae	P62260	14-3-3 protein epsilon	enzyme regulator activity; protein binding
Sept5	Q9JJM9	Septin-5	catalytic activity; nucleotide binding; protein binding
Mog	Q63345	Myelin-oligodendrocyte glycoprotein	protein binding; receptor activity; signal transducer activity
Snca	P37377	Alpha-synuclein	enzyme regulator activity; metal ion binding; protein binding; transporter activity
Fam54b	Q5XII9	Fam54b	unknown
Hsph1	Q66HA8	Heat shock protein 105 kDa	nucleotide binding; protein binding
Pygb	P53534	Glycogen phosphorylase, brain form	catalytic activity; protein binding
Lasp1	Q99MZ8	LIM and SH3 domain protein 1	metal ion binding; protein binding; transporter activity
Cndp2	Q6Q0N1	Cytosolic non-specific dipeptidase 2	carboxypeptidase activity; metal ion binding; metallopeptidase activity; tripeptidase activity
Camk2d	P15791	Calcium/calmodulin-dependent protein kinase type II subunit delta	catalytic activity; nucleotide binding; protein binding
Acaa1a	P21775	3-ketoacyl-CoA thiolase A, peroxisomal	catalytic activity
Ppp1r1b	Q6J4I0	Protein phosphatase 1 regulatory subunit 1B	enzyme regulator activity; protein binding

Fig. 2 (A) Differential binding of proteins to *N*-hydroxysuccinimide-derived sepharose with no peptide (beads alone) or immobilised peptides **1**, **2** and **3**. Proteins are arranged in descending order with respect to their ability to bind *O*-GlcNAcylated peptide **2**. *A combined result is presented for AP180 related proteins Vamp1 and Vamp2. Results are the average intensity from three independent experiments ± standard error of the mean. (B) Gene names, UniProtKB accession number, protein name and UniProtKB gene ontology molecular function terms for each of the proteins detected.

Apart from Vamp2, this study revealed altered binding of the *O*-GlcNAc and *O*-GlcNAc-6-phosphate modified AP180 peptides to proteins with various other physiological functions. Future work will focus on binding studies using longer sequences of modified AP180 to validate these results. Importantly, the Fmoc-protected glycosylamino acid building block **9**

prepared in this work will have wider utility. Specifically, the building block can be used in the synthesis of β-*O*-GlcNAc-6-phosphate-derived AP180 peptides as standards for quantitative proteomics analysis and antigens to generate modification-specific antibodies. Therefore, this work is the first step in the production of important tools for *in situ* detection which will aid in determining the functional role of β-*O*-GlcNAc-6-phosphate both in SVE and potentially in other roles in biology.

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