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New Directions in Glycoprotein Engineering

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Abstract—Semisynthetic protein engineering techniques can be employed to overcome classic bottlenecks in the total synthesis or recombinant production of proteins bearing natural or unnatural post-translational modifications. Glycoproteins are particularly suited to semisynthesis as homogeneous samples for biological analyses are not readily available using traditional recombinant techniques. Here we report the use of expressed protein ligation for the construction of semi-synthetic glycoproteins, which are assembled when recombinantly derived peptide α-thioesters are covalently joined with synthetic glycopeptides with clustered O-linked glycans by native chemical ligation. We demonstrate how this approach may be employed for the construction of glycoproteins bearing mucin-like domains.

Introduction

The measure of understanding associated with the biological role of glycoconjugates has traditionally fallen far behind that of proteins and nucleic acids. It is, however, now well established that protein glycosylation can be a vital co/post-translational modification for the normal growth and development of organisms. This is compounded by the knowledge that the glycan moiety of glycoproteins is implicated in an increasing number of important biological processes from correct protein folding and secretion to cell–cell recognition.[1–4] Understanding how and why these highly specific interactions come about has always been hindered by the difficulties associated with the 'micro-heterogeneity' of glycoproteins.[5,6] The structural characterization of components in a mixture composed of structurally similar biomolecules has stretched the abilities of separation and analytical techniques to their limits and is often prohibitively labor-intensive. These difficulties, in addition to the fact that protein glycosylation is not under direct genetic control, have been a driving force for chemists to develop new methods for glycoprotein synthesis. As a result of advances in carbohydrate chemistry, the synthesis of large glycans is now becoming more accessible[7–9] but it is still far from routine and its union with peptide synthesis less routine still, owing to increased protecting group demands and poor α/β selectivity in glycosidation reactions.[10] The overall result is that we presently lack the technology to rapidly assemble complex glycoproteins and therefore the ability to prepare homogeneous standard compounds of known structure for biological analyses, an obstacle we hope to overcome through the fusion of novel chemical and biological methods.

One might be forgiven for concluding that nature has conspired to make life for the glycobiologist as difficult as possible. We know that prokaryotes can be employed to express large quantities of recombinant proteins, yet they do not glycosylate them.[11,12] Mammalian cells, on the other hand, can be employed to produce modest quantities of recombinant glycoproteins and these have been exploited for the production of therapeutics. Large scale preparations are extremely costly[13] and the products usually exist as heterogeneous (though rigorously characterized) mixtures of glycoforms.[5] Wong and co-workers, however, have shown how proteins which are obtained as heterogeneous mixtures can be 'remodeled' in-vitro by the action of glycosidases and glycosyltransferases to remove the native glycans and build up known structures in their place.[14] This approach may require access to specific glycosidases and glycosyltransferases (many of which are not yet commercially available) and the acquisition of costly glycosyl nucleotide donors. Consequently, efforts to alter metabolic processes which can add novel glycans through co-expression of non-native glycosyltransferases or delete glycans through transferase or epimerase disruption, inhibition or gene knock-out have also received a great deal of attention and have been recently reviewed.[15–20]

Glycoform engineering on the surface of living cells

Recently we have shown that through the addition of metabolic precursors of sialic acid: *N*-levulinoylmannosamine[21] (ManLev) and *N*-azidoacetylmannosamine[22] (ManAz), cell surface glycoproteins can be made to display ketones and

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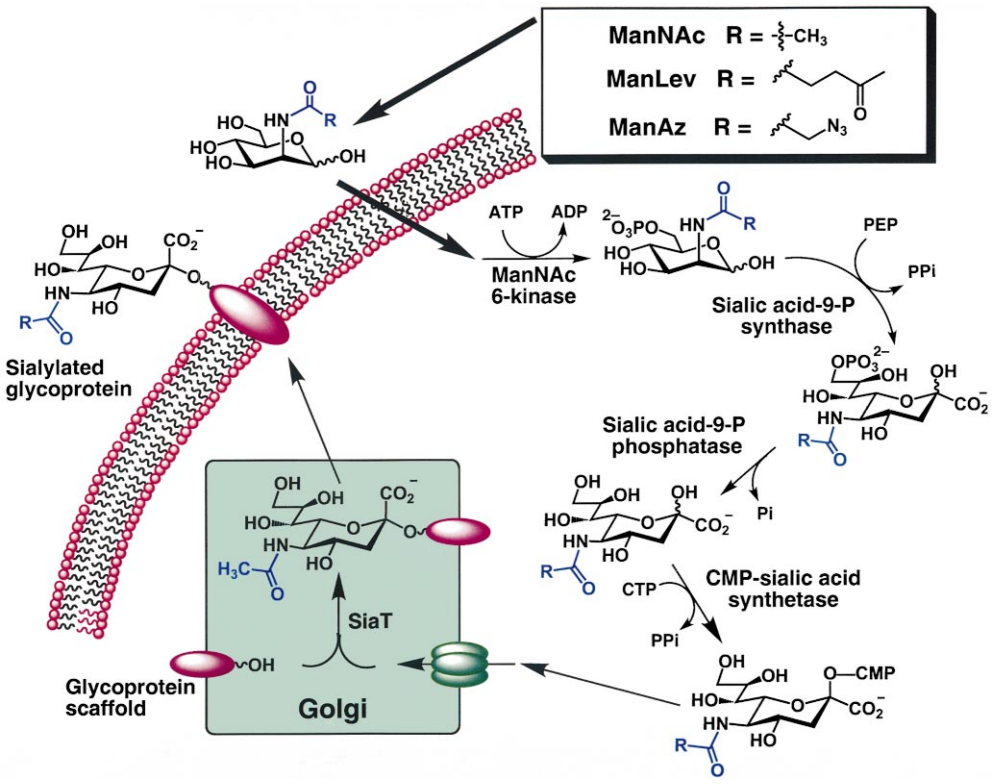

Figure 1. Unnatural analogs of *N*-acetylmannosamine (ManNAc) bearing ketones (ManLev) or azides (ManAz) are metabolized to unnatural sialic acids on cell surface glycoproteins, allowing chemical modification of cell surface oligosaccharides.

azides (Fig. 1) which may ultimately be extended by selective covalent reactions.[23] In the case of ketones, complementary nucleophiles such as hydrazides and aminooxy groups can react in the cellular environment to form stable adducts and this has found many applications, including potential tumor diagnostics.[24,25] The azide on the other hand has been shown to undergo Staudinger chemistry.[22] From these reactive handles on cells it is then possible to build new epitopes on endogenous glycoconjugates to provide new cell surface properties.[26] This technology,

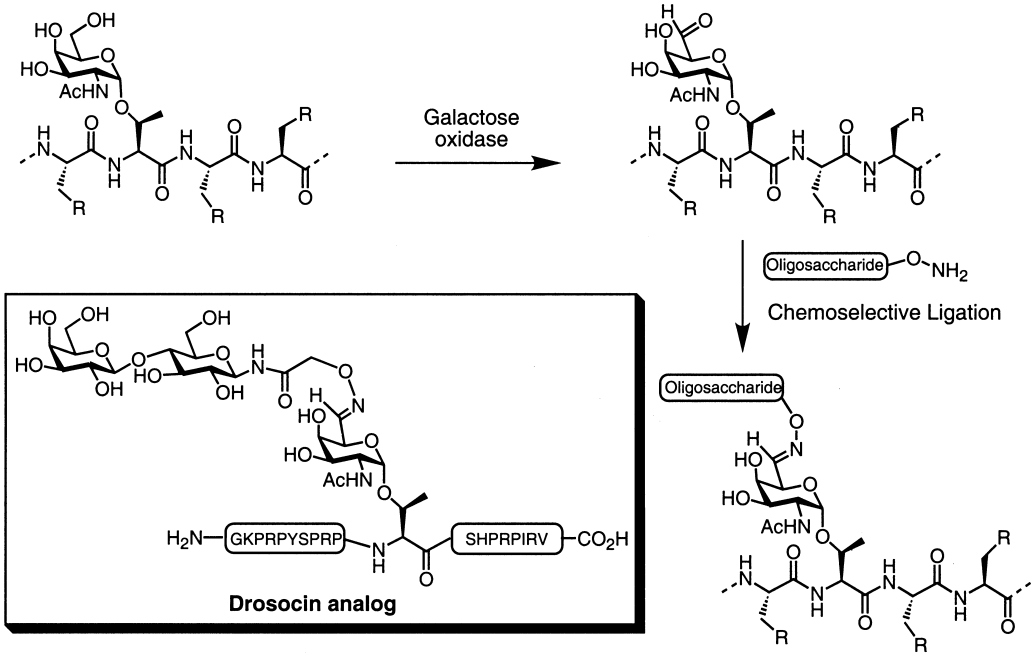

Figure 2. Galactose oxidase-mediated oxidation of the 6-hydroxyl group of the proximal *N*-acetylgalactosamine residue allows for structural elaboration with synthetic aminooxy glycosides. An analog of an antimicrobial glycopeptide, drosocin, was prepared using this method.

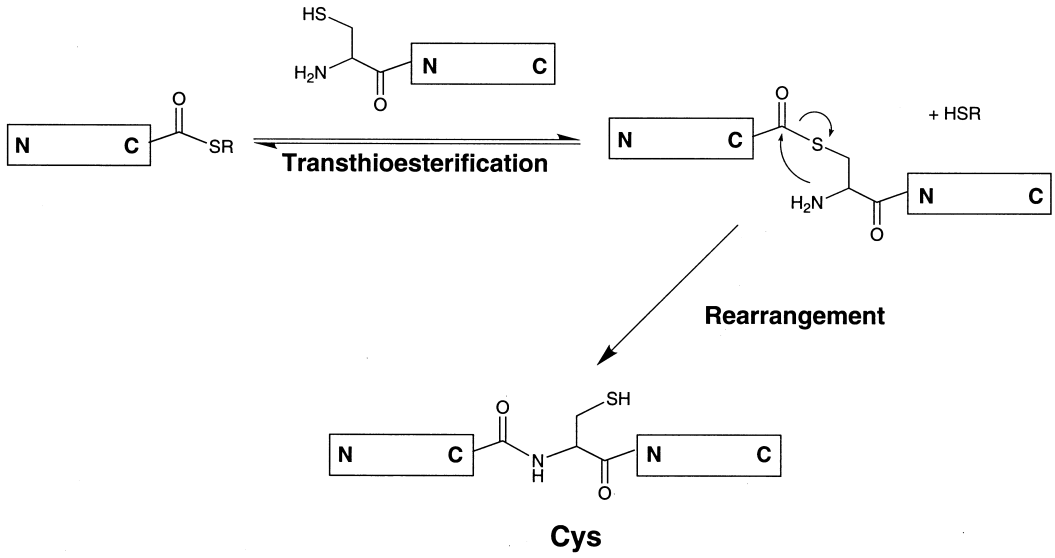

Figure 3. The mechanism of native chemical ligation. A peptide α-thioester undergoes a reversible transthioesterification reaction with a second peptide bearing an N-terminal cysteine residue. A spontaneous rearrangement (S→N acyl shift) affords a native peptide bond.

though useful, is non-specific with respect to any particular glycoprotein on the cell surface and is therefore of little general synthetic utility.

Total and semisynthesis of glycoproteins and mimetics

Although they lack the protein glycosylation machinery of mammalian cells, prokaryotes can be employed to express large quantities of recombinant proteins, and, not surprisingly, attempts to synthetically modify such proteins with glycans have received great attention.[27] Most frequently such 'neoglycoproteins' are realized through the coupling of activated saccharides non-specifically to the side chains of lysine or cysteine residues resulting in unnatural sugar-peptide linkages.[28,29] Neoglycoproteins have been used to present carbohydrate epitopes in a polyvalent fashion, thereby mimicking the natural polyvalent display of carbohydrate antigens in living organisms, and are now being employed in carbohydrate-based vaccines.[30–32]

The advent of solid-phase chemistry by Merrifield in 1963 revolutionized the way in which large biomolecules could be prepared.[33] The use of solid-phase chemistry for the preparation of peptides and proteins has become commonplace. Although the solid-phase synthesis of small glycopeptides has been realized[34–46] it is still far from routine and suffers from the previously mentioned drawbacks associated with carbohydrate and glycopeptide chemistry. Furthermore, the convergent union of oligosaccharides with a peptide scaffold has only been realized for N-linked glycopeptides; O-linked glycopeptides are generally prepared through the incorporation of glycosylated building blocks in stepwise solid-phase peptide synthesis (SPPS) as a consequence of the difficulties associated with the formation of the glycosidic linkage. We have previously shown that this problem may be surmounted through another chemoselective ligation approach.[42] Preparing synthetic glycans as aminooxy glycosides allows them to be coupled selectively to carbohydrate structures bearing aldehyde functionalities which are in turn generated through the action of galactose oxidase on the 6-hydroxyl group of *N*-acetylgalactosamine (Fig. 2). Unlike most other neoglycoprotein preparations, the natural carbohydrate-peptide linkage is maintained, although an internal glycosidic bond is replaced with an unnatural oxime linkage.

Additionally, stepwise peptide synthesis becomes inefficient for peptides greater than 50 amino acid residues in length and few groups have prepared proteins greater than 100 residues in length by this method.[47] The fact remains that the majority of synthetic glycopeptides prepared to date are small (20 or so residues) and most biologically significant proteins and glycoproteins are considerably larger. In order to overcome these obstacles, elegant approaches have been devised which enable the glycopeptide chemist to take advantage of recent developments in convergent protein synthesis, particularly methods for peptide fragment coupling such as native chemical ligation (Fig. 3).[48,49]

Native chemical ligation for the synthesis of small glycoproteins

Native chemical ligation involves the coupling of two unprotected peptide fragments with a C-terminal thioester on one fragment and an N-terminal cysteine residue on the other (Fig. 3). The two moieties combine reversibly through a transthioesterification reaction and then spontaneously rearrange to form a native peptide bond. Originally, thioesters were prepared using traditional *tert*-butyloxycarbonyl (Boc)-based solid-phase chemistry (on a thioester resin) which was incompatible with the established 9-fluorenylmethoxycarbonyl (Fmoc)-based synthesis of glycopeptides.[40,41] Most importantly, the thioesters were not stable to the basic conditions of Fmoc SPPS and the acid sensitive glycosidic linkages were unstable to the harshly acidic, repetitive deprotection conditions of traditional Boc-based SPPS. This conflict was elegantly solved using Ellman's modification of Kenner's sulfonamide safety catch linker (Fig. 4).[50] This allowed a glycopeptide to be prepared using standard Fmoc SPPS, with the final amino acid

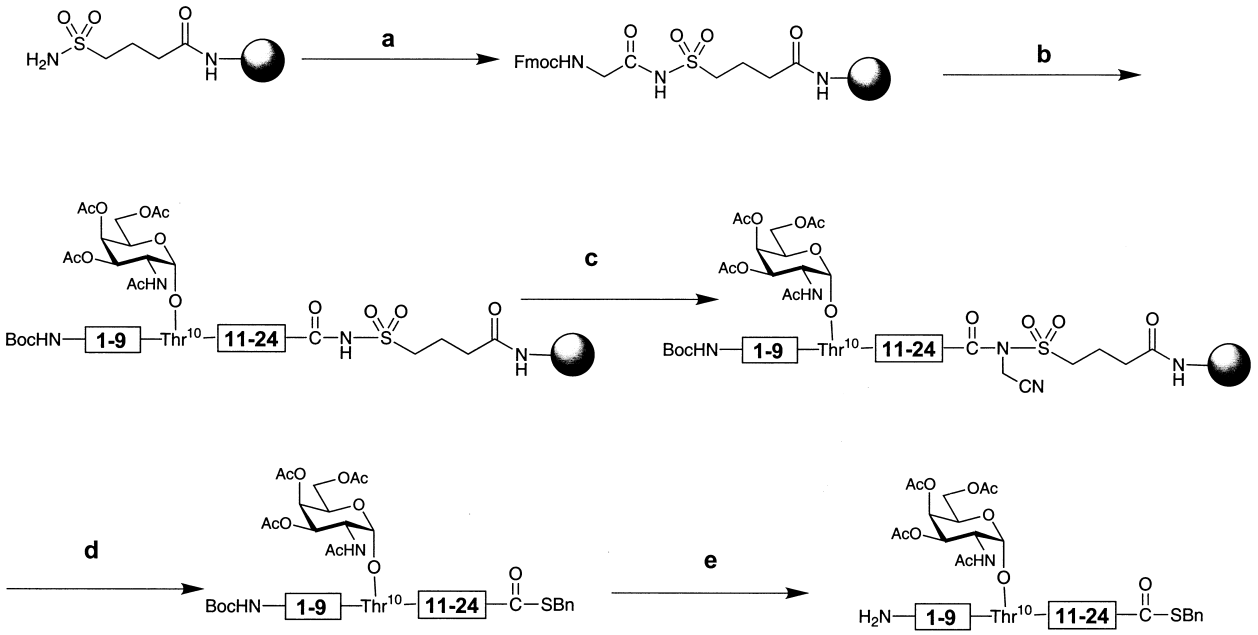

Figure 4. Chemical synthesis of peptide α-thioesters for the total synthesis of diptericin. (a) Fmoc-Gly-OH, pyBOP, DIEA, DMF, −20°C, 8 h (double coupling), 97%; (b) Fmoc SPPS; (c) ICH_2CN/DIEA, NMP, 24 h; (d) BnSH, THF, 24 h; (e) TFA.[51]

residue coupled in Boc-protected form. The alkylsulfonamide linker could then be activated by alkylation using an α-halo acetonitrile, which then facilitates the attack of sulfur nucleophiles such as thiophenol or benzyl mercaptan. The resulting thioester can be purified and employed in native chemical ligation reactions. This approach was successfully used in the preparation of the 82-residue glycoprotein diptericin (Fig. 5).[51]

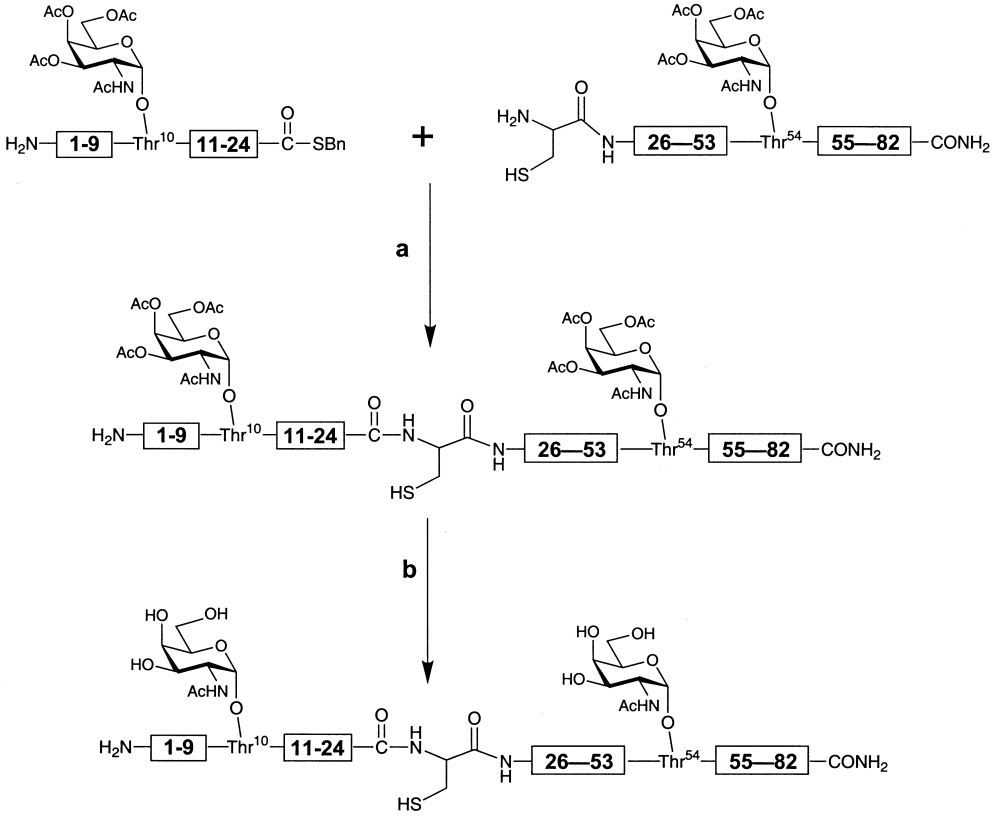

Figure 5. Native chemical ligation approach to the synthesis of diptericin. (a) 6 M guanidine·HCl/ 0.1 M phosphate; pH 7.5, 4% thiophenol, rt, 24 h, 55%; (b) NH_2NH_2/ DTT, 53%.[51]

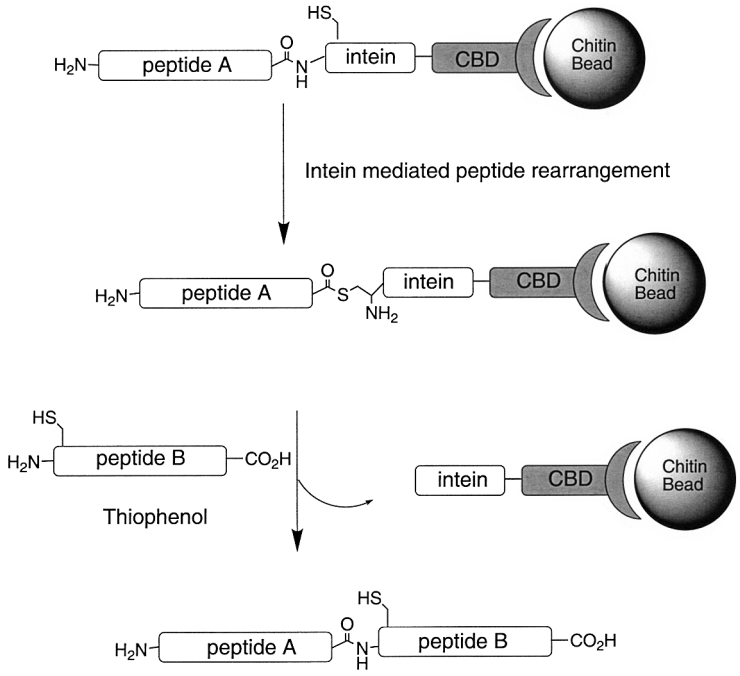

Figure 6. Expressed protein ligation. The commercially available IMPACT CN expression system allows for the bacterial production of target proteins fused to an intein,[53] followed by a chitin binding domain (CBD) which facilitates purification on chitin beads. The intein-mediated peptide cleavage reaction produces recombinant peptide α-thioesters, which can be utilized in native chemical ligation reactions.[53]

Expressed protein ligation: a versatile method for glycoprotein synthesis

Given the limitations associated with solid-phase peptide synthesis, and regardless of their abatement with native chemical ligation, proteins larger than 20 KDa in size are still difficult to make synthetically. Expressed protein ligation, a method for generating recombinant peptide-thioesters based on the natural phenomenon of protein splicing,[52] has been used to great effect by Muir

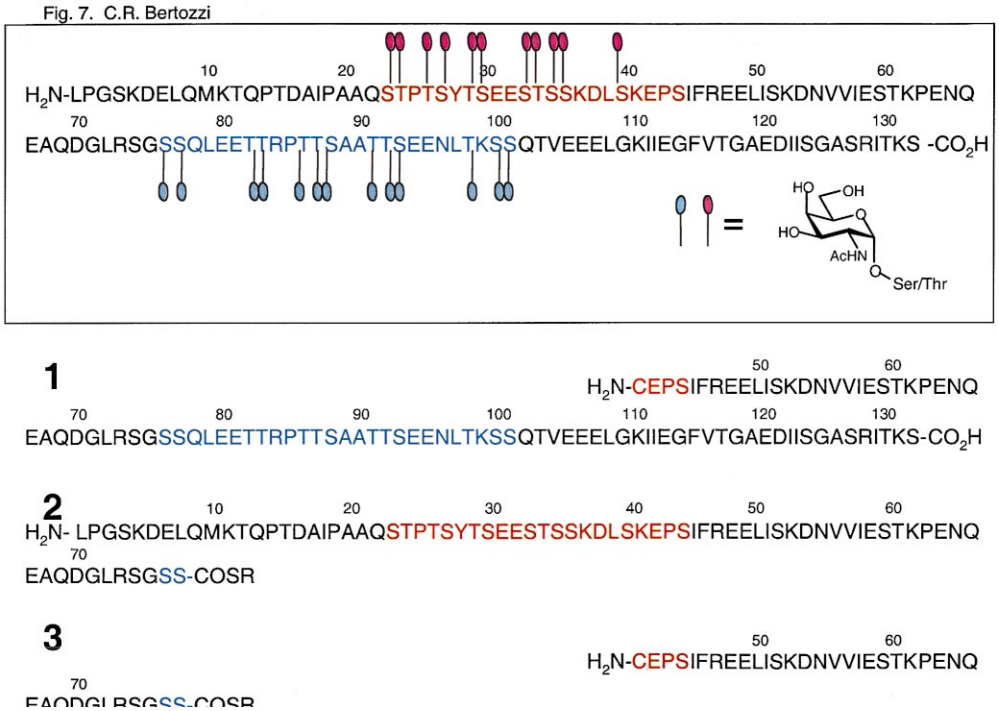

Figure 7. The primary sequence of GlyCAM-1 showing putative sites of glycosylation which comprise mucin-like domains (boxed). Using suitable PCR primers we can generate GlyCAM-1 fragments which, after bacterial expression, allow for the semi-synthesis of GlyCAM-1 lacking the N-terminal, C-terminal or both mucin domains (fragments **1**, **2**, and **3** respectively).

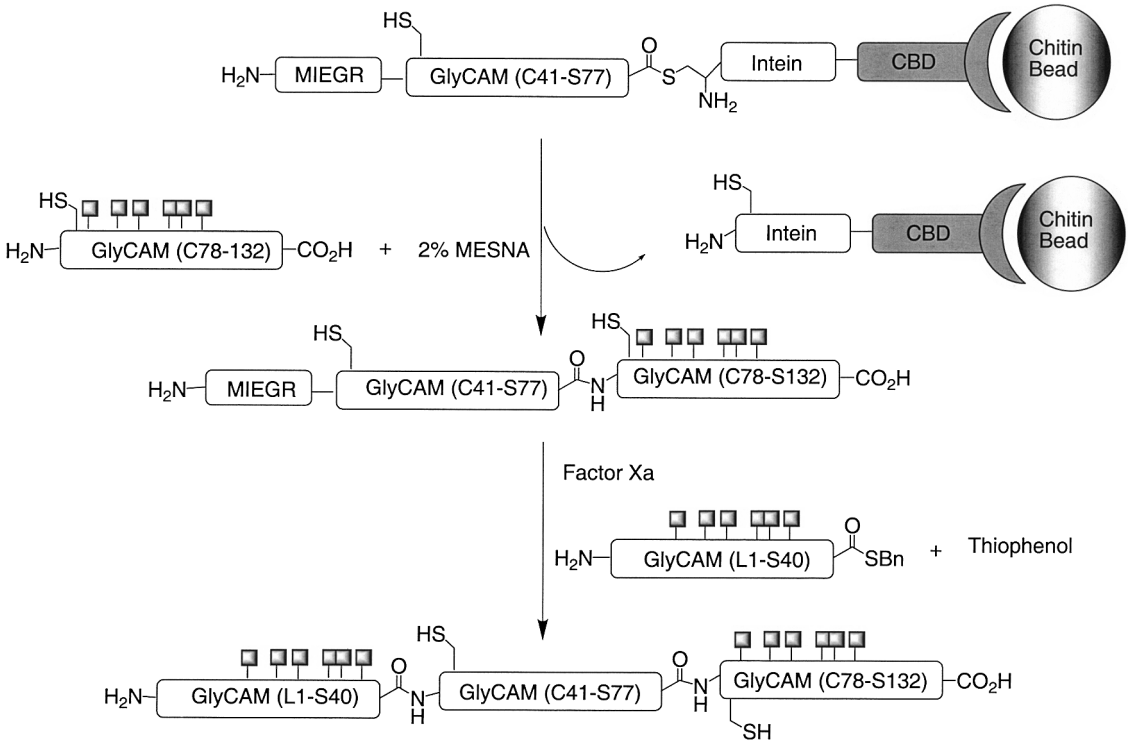

Figure 8. Tandem ligation approach for the construction of fully glycosylated GlyCAM-1.

and co-workers for the synthesis of large biologically active proteins (Fig. 6).[53] These researchers have shown how a small peptide containing a phosphotyrosine motif could be ligated to the remainder of the Csk protein backbone produced by recombinant means.[54] Most recently, Wong and co-workers have indicated that glycoprotein synthesis may be possible using synthetic peptides bearing N-linked glycans employing this approach.[55] Given our interest in

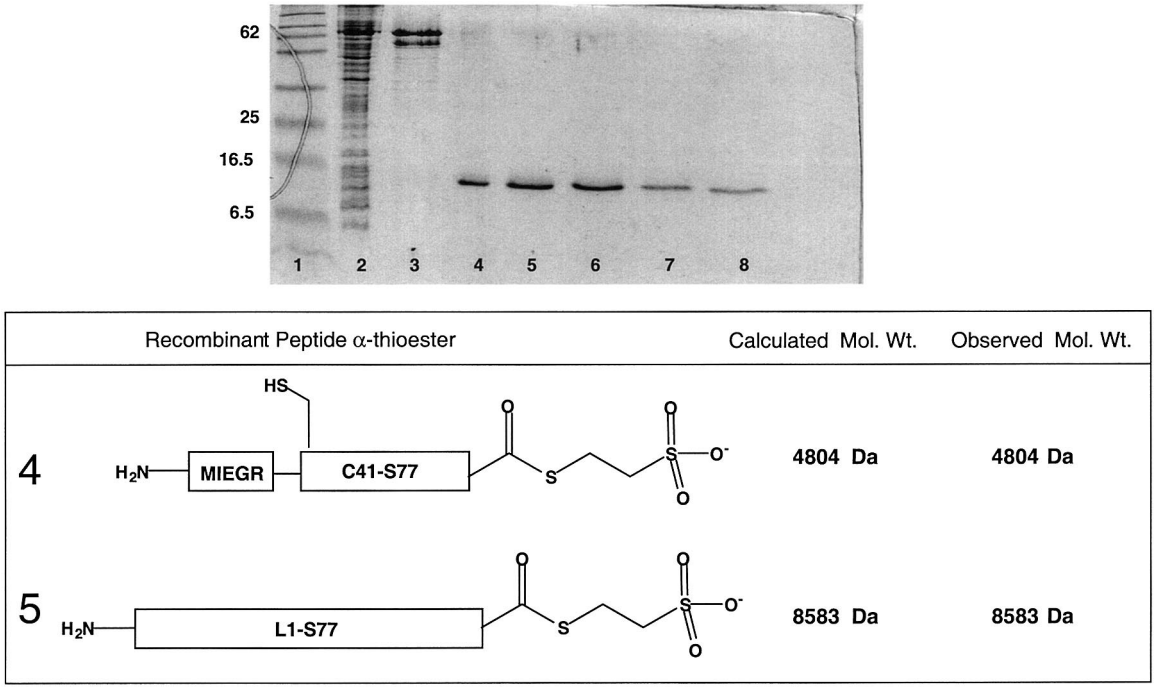

Figure 9. SDS-PAGE analysis of an expressed GlyCAM-1 fragment. Lane 1, molecular weight markers with masses shown in KDa. Lane 2, the prepared cell-free extract showing over-expression of the N-terminal fragment of GlyCAM-1, residues1–77 (**2**), fused to intein-CBD with an approximate molecular weight of 62 KDa. Lane 3, proteins bound to the chitin beads after 24 h cleavage with 2% MESNA. Lanes 4–8, eluted 0.5 mL fractions containing thioester **5**. We used this method to characterize thioesters **4** and **5**.

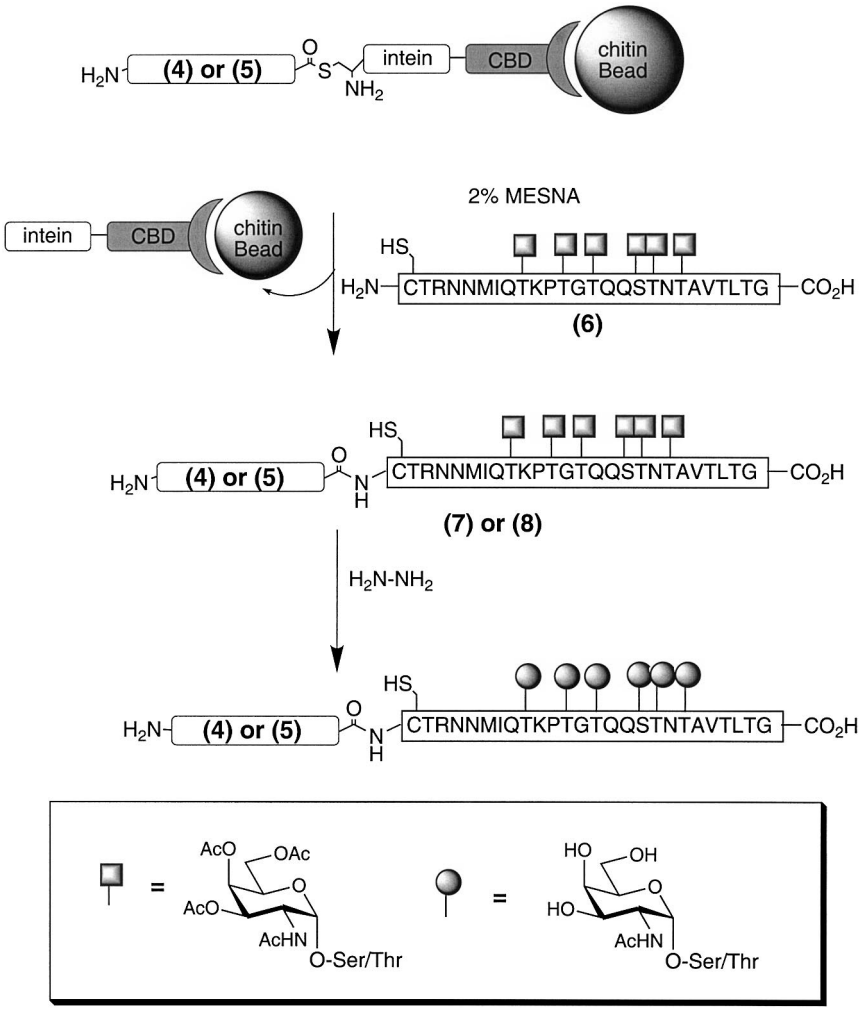

Figure 10. Ligation reactions between expressed thioesters **4** or **5** and synthetic glycopeptide **6** afford ligated chimeric glycoproteins **7** and **8**.

O-linked glycoproteins involved in the inflammatory response,[56,57] we aimed to apply expressed protein ligation to the preparation of glycoproteins bearing mucin-like domains (ie. clustered glycans characterized by *N*-acetylgalactosamine (GalNAc) residues α-O-linked to the hydroxyl groups of serine or threonine residues of the protein backbone). If successful, then the ability to readily prepare glycoproteins containing well defined O-(or N-)linked glycans represents a major advance for the study of glycobiology.

As our glycoprotein target we have chosen GlyCAM-1 (Fig. 7). First characterized in mouse, GlyCAM-1 is a secreted glycoprotein which has extensive O-linked glycosylation and is a known endothelial-derived ligand for L-selectin[58] thought to be involved in leukocyte adhesion to inflamed endothelium. The carbohydrate moieties have been characterized[59] and are known to contain a 6-sulfo sialyl Lewis x motif (where sulfation enhances its L-selectin binding affinity) which has been synthesized recently by Kiso and co-workers.[60] We ultimately aim to elucidate the importance of its mucin domains (residues 22–43 and residues 73–102, Fig. 7-boxed) for biological activity. Towards this end, we have undertaken its semi-synthesis using expressed protein ligation together with solid-phase glycopeptide synthesis. Our proposed strategy for the construction of fully glycosylated GlyCAM-1 is shown in Fig 8.

Results and Discussion

To fully investigate the function of the O-linked mucin domains in GlyCAM-1 we have prepared a number of DNA constructs that will allow us to introduce the N-terminal mucin domain (residues 1–40, Fig. 7), and the C-terminal mucin domain (contained within residues 78–132) either independently, or in concert (through the tandem native chemical ligation approach in Fig. 8). Such an approach allows us to evaluate the effects of glycan structure on function, and the effects of glycan site-occupancy on function as well.

Herein we discuss the expression of those fragments (**2** and **3**, Fig. 7) which are recombinantly-derived C-terminal thioester peptides to be used in the synthesis of glycoforms of GlyCAM-1. Our initial investigations have employed a synthetic 'model' mucin domain which was coupled with such expressed fragments by native chemical ligation.

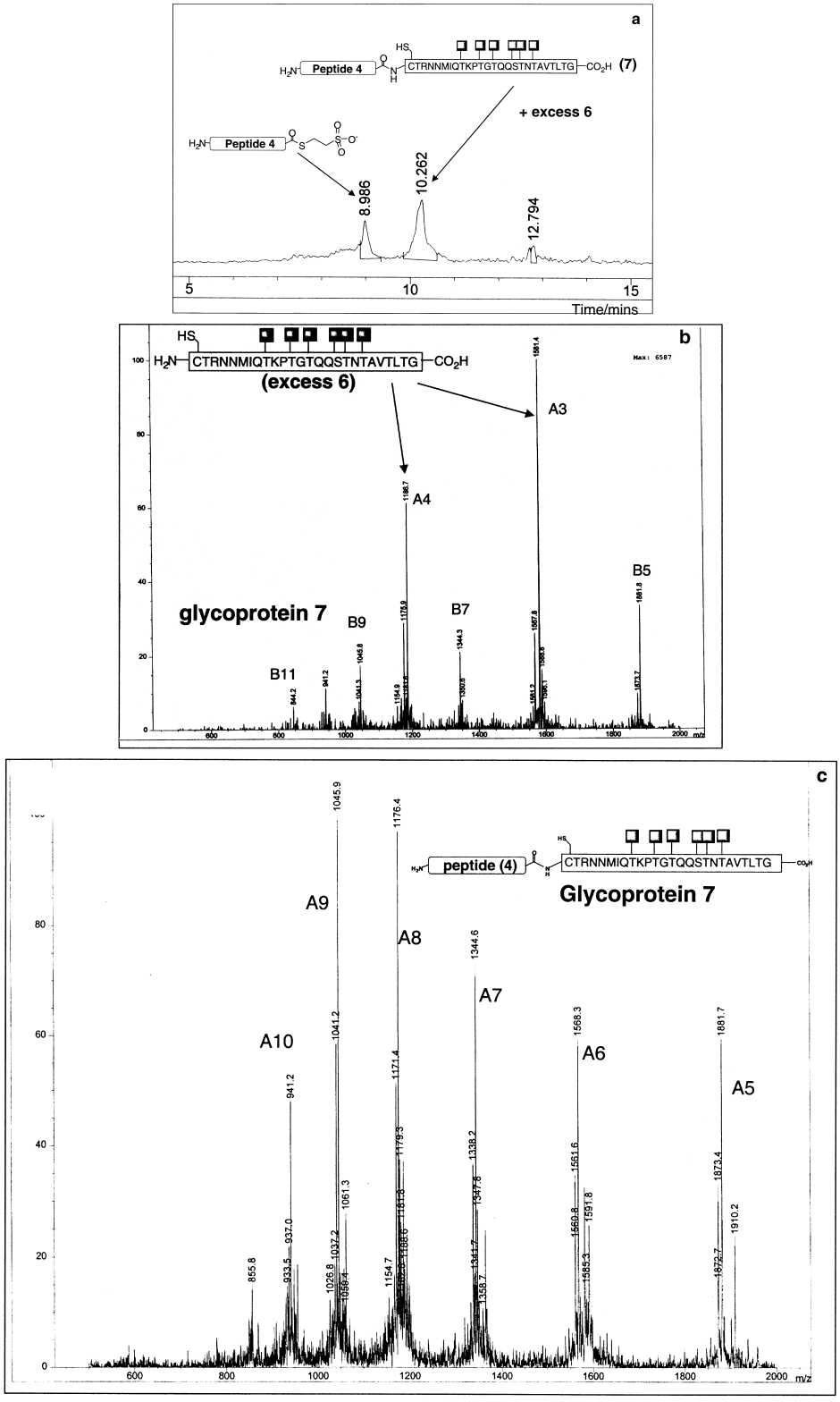

Figure 11. Analysis of the ligation reaction and products. (a) LC-MS analysis showing unreacted thioester **4** eluting after approximately 9 min and the glycopeptide product co-eluting with starting material (**6**) at 10.3 min. (b) LC-electrospray MS data depicting the excess glycopeptide **6** co-eluting with the glycoprotein product **7**. (c) The product glycoprotein (calculated molecular weight=9404 Da, observed molecular weight=9404 Da) after HPLC purification.

Expression vector construction and protein expression

Gene fragments were PCR amplified and sub-cloned using *Nde*I/*Sap*I (encoding no vector-derived amino acids) into the commercially available intein fusion expression vector pTYB-1.[61] After purification on chitin beads, the peptide thioesters were cleaved with 2% w/v mercaptoethane sulfonic acid (MESNA) and characterized by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass analysis (Fig. 9). In the case of peptide

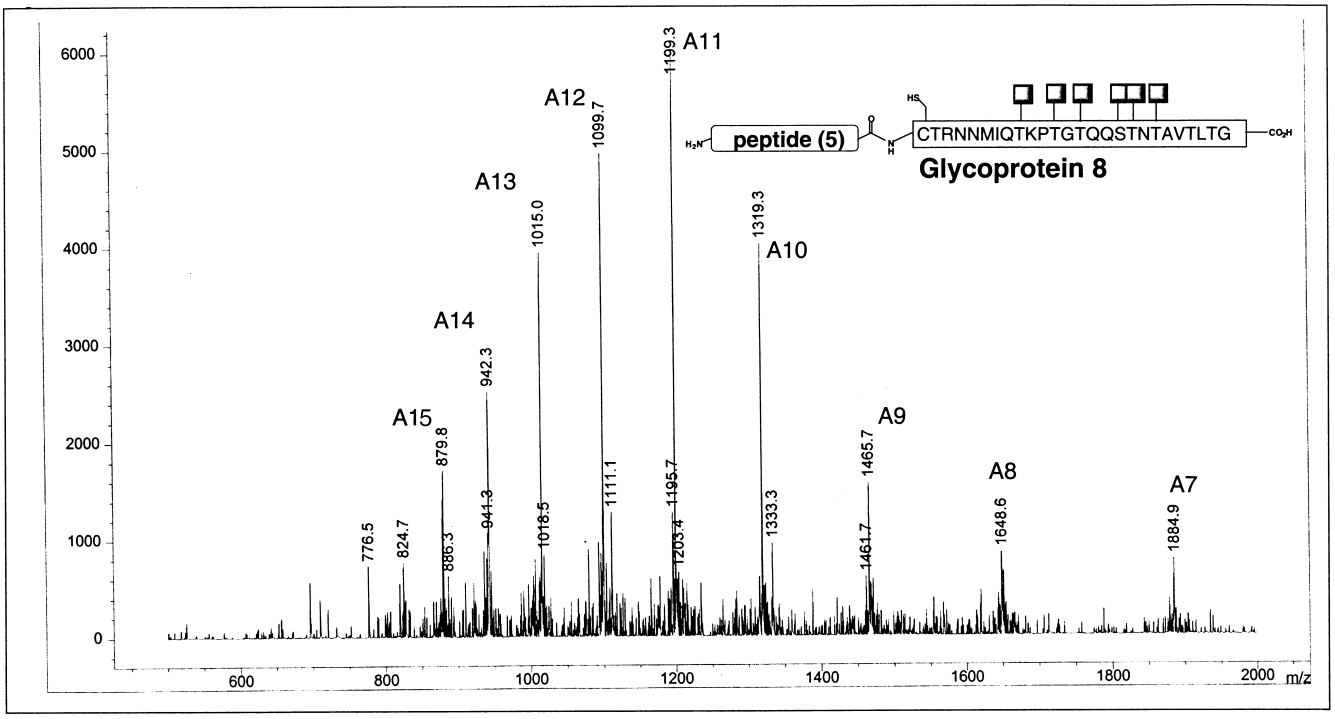

Figure 12. HPLC purified glycoprotein product **8** (calculated mol. wt.=13184 Da, observed mol. wt.=13183 Da) from the ligation of thioester **5** with glycopeptide **6**.

thioester **5** (the MESNA thioester derivative of fragment **2**), the thioester was the only product observed by LC-MS after cleavage, whereas **4** suffered approximately 5% hydrolysis to the free acid during purification. Peptide cleavage was highly efficient using 50 mM DTT as well and milligram quantities of each thioester was obtained (determined by SDS-PAGE).

Glycopeptide synthesis

Peracetylated *N*-acetylgalactosamine (GalNAc) α-O-linked to Fmoc-protected serine or threonine were generated using published procedures.[52,62] The glycopeptide **6** (Fig. 10) was prepared using standard Fmoc chemistry employing DCC/HOBt or HBTU/HOBt as coupling reagents. This 'model' mucin domain corresponded to a putative mucin-like stretch of human lymphotactin (residues 68–93), another glycoprotein under study within our lab.[63] In each coupling reaction, five equivalents of Fmoc-GalNAc serine or threonine were used and the progress was monitored by LC-MS. Once the synthesis was complete, the glycopeptide was cleaved from the resin using reagent K (TFA/H_2O/phenol/thioanisole/ethanedithiol),[64] precipitated with ether, and purified by reversed-phase HPLC.

Ligation reactions (Fig. 10)

GlyCAM-1 fragment **3** (Fig. 7) expressed as its intein-CBD fusion was immobilized on chitin beads and washed with buffer (200 mM sodium phosphate, 100 mM NaCl; pH 8.0, 30.0 mL) and again with buffer containing 2% MESNA (5.0 mL) to produce the thioester **4** in-situ. This was immediately followed by buffer containing 2% MESNA and an excess of model glycopeptide **6** (Fig. 9). After 24–48 h the buffer was eluted from the column and 0.5 mL fractions were collected. Each fraction was analyzed by LC-MS (Fig. 11) and after 48 h, the reaction was still incomplete. This was demonstrated by the presence of unreacted thioester (and some free acid) which eluted from the column after 8.98 min (Fig. 11a). After 10.26 min the ligated glycoprotein product (**7**) was found to co-elute with the excess glycopeptide starting material **(6)** by LC-MS (Fig. 11b). We had resolved to co-purify both **6** and **7** (while separating them from unreacted thioester **4**) by reversed-phase HPLC and then to separate them from each other after the carbohydrate residues were deacetylated using hydrazine (this was shown to be effective when the ligation reaction mixture was treated directly with hydrazine). Chitin column fractions were pooled and concentrated using a centricon (millipore, 3 KDa molecular weight cutoff) at 4°C to a final volume of 0.5 mL. After concentration, the glycoprotein product was purified using semi-preparative reversed-phase HPLC. As it turned out, both species (product **7** and starting material **6**, Fig. 11b) were separated efficiently on a semi-preparative reversed-phase column. The HPLC purified glycoprotein (Fig. 11c) was stored as a lyophilized solid at −20°C.

In a further ligation reaction, the N-terminal fragment of GlyCAM-1 (residues 1–77, **(5)**) was ligated to the same mucin domain **(6)** as previous. Again, the ligation reaction appeared to be highly efficient yet was not quantitative, despite the large excess of glycopeptide added. After HPLC purification of the glycoprotein product **8** (Fig. 12), it was readily deprotected by the addition of hydrazine hydrate to a final concentration of 5% v/v (Fig. 10). Deprotected glycoproteins were again purified by HPLC and gave masses in agreement with calculated values.

Conclusions and Future Work

Using a model glycopeptide fragment **(6)** containing a mucin domain (six α-O-linked GalNAc residues), we prepared large glycoproteins (greater than 12 KDa) on a semi-preparative (5 mg) scale employing the commercially available pTYB-1 expression vector for the preparation C-terminal thioester peptides suitable for use in expressed protein ligation. An additional advantage of this expression system is that no vector-derived amino acids need be encoded. Having access to milligram quantities of homogeneously glycosylated glycoproteins should simplify structural and functional determinations. Our next goal is to utilize this model system to study the extension of the glycan moiety using established methodology (see Fig. 2). Furthermore, the synthesis of the native GlyCAM-1 mucin domains (Fig. 8) is nearing completion. Such homogeneous glycoprotein products will give us new insights into the functional role of the mucin domains.

Experimental

General

Peptide synthesis was carried out on an Applied Biosystems model 431A peptide synthesizer using pre-loaded Wang resin and Fmoc amino acids from Novabiochem. The IMPACT CN system and all other molecular biology reagents were obtained from New England Biolabs. Oligonucleotide primers were obtained fully desalted and deprotected from Genosys. PCR was carried out on a Perkin Elmer Cetus thermal cycler. Mass spectra were obtained on a Hewlett Packard LC-MSD1100 series electrospray LC-MS. LC-MS was performed using a Zorbax C18 LC-MS column (2.1×50 mm) and a gradient of 10–90% acetonitrile containing 0.1% TFA over 15 min (flow rate of 0.3 mL/min). Semi-preparative HPLC was performed using a Rainin DYNAMAX C18 column and a gradient of 10–80% acetonitrile containing 0.1% TFA over 50 min (flow rate of 3.0 mL/min). All other chemical reagents were obtained form Aldrich.

Solid-phase glycopeptide synthesis

Solid-phase peptide synthesis was carried out in an automated fashion using ten equivalents of Fmoc amino acid per coupling reaction and DCC/HOBt as coupling reagents. Coupling time was 2 h. Glycoamino acids were coupled manually using five equivalents of Fmoc-Ser($(AcO)_3$GalNAc)-OH or Fmoc-Thr($(AcO)_3$GalNAc)-OH and HBTU/HOBt as coupling reagents. On average the coupling time was 3 h and the reaction progress was monitored by LC-MS. This procedure was used for the synthesis of the model mucin domain **(6)**: H_2N-CTRNNMIQT($(AcO)_3$GalNAc)KPT($(AcO)_3$GalNAc)GT($(AcO)_3$GalNAc)QQS($(AcO)_3$GalNAc)T($(AcO)_3$GalNAc)NT($(AcO)_3$GalNAc)AVTLTG-CO_2H. Calculated molecular weight=4743 Da, observed molecular weight=4742 Da.

Bacterial protein expression

Fragments of GlyCAM-1 were PCR-amplified using suitable primers, allowing them to be ligated using restriction endonucleases *Nde*I/*Sap*I into the IMPACT CN expression vector pTYB-1 (New England Biolabs). Protein expression and purification were carried out essentially according to the manufacturers instructions: Bacterial cultures (500 mL) were grow to OD_{600}=0.6 and induced by the addition of IPTG to a final concentration of 0.5 mM. Protein expression was continued for 6 h at 25–30°C after which time the cells were harvested by centrifugation (10000×G, 10 min). The harvested cells were then resuspended in cell lysis buffer (20 mM Tris·HCl; pH 8.0, 500 mM NaCl, 0.1% Tween 20, 0.1 mM TCEP and 0.001% PMSF, 30 mL) and lysed by sonication (3×30 s) on ice. The cell-free extract, containing soluble GlyCAM-1-intein-CBD fusion, was obtained after centrifugation (10000×G, 15 min) to remove cellular debris. The extract could be stored at −20°C prior to purification.

Glycopeptide ligation

The cell free extract was loaded onto 1.5 mL of chitin beads equilibrated with cell lysis buffer (10.0 mL). The beads were then washed with phosphate buffer (200 mM sodium phosphate; pH 8.0, 100 mM NaCl, 50 mL), followed by phosphate buffer containing 2% w/v MESNA (5.0 mL). The column was allowed to run dry and then plugged. Finally, phosphate buffer containing 2% w/v MESNA and the synthetic peptide **6** was applied (2.0 mL) and the contents mixed by gentle pipetting. After 24–48 h the plug was removed and 0.5 mL fractions were collected and analyzed by SDS-PAGE and LC-MS.

Glycoprotein deacetylation

The purified glycoprotein was then dissolved in phosphate buffer and treated with hydrazine hydrate to a final concentration of 5% v/v. After 16 h, the glycoprotein was purified by semi-preparative reversed-phase HPLC, lyophilized and stored at −20°C.

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