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Use of carbohydrates and peptides in studies of adhesion of pathogenic bacteria and in efforts to generate carbohydrate-specific T cells

J. Kihlberg and G. Magnusson

Organic Chemistry 2, Chemical Center, The Lund Institute of Technology, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

Abstract: The recognition of cell surface carbohydrates by *E. coli* and *S. suis* has been investigated. Use of synthetic oligosaccharides and their deoxy- and deoxyfluoro derivatives as inhibitors of the bacterial adhesins revealed that *E. coli* and *S. suis* were hydrogen bonded to different epitopes on the disaccharide galabiose. In addition, synthetic peptides were identified that inhibit the chaperone PapD which is required for assembly of protein subunits into the *E. coli* P pilus. Both adhesin-receptor interactions and subunit-chaperone interactions constitute targets for design of novel antibiotics. The T cell response to derivatives of immunogenic peptides that carry carbohydrate haptens has also been studied and carbohydrate-specific helper T cells were obtained on immunization of mice. An enhanced immune response against carbohydrate antigens may be useful in treatment of infectious disease and cancer.

INTRODUCTION

All animal cells contain glycosphingolipids which have the lipid (ceramide) moieties embedded in the cell membrane and the hydrophilic carbohydrate moieties protruding into the surrounding medium (1). Glycosphingolipids are thus ideally located to participate in interactions between the cell and its environment and play important roles in processes such as cell growth and differentiation. In addition, viruses, bacteria, and bacterial toxins use the protruding carbohydrate moieties as specific sites of attachment in infectious processes. Glycolipids belonging to the globoseries (2) and to the P blood group system (3) all contain a β-D-galabiose [α-D-Gal*p*-(1→4)-β-D-Gal*p*] moiety (Fig. 1) which serves as ligand for protein receptors of the bacteria *Escherichia coli* (4, 5) and *Streptococcus suis* (6), as well as the bacterial toxins verotoxin from *E. coli* (7) and Shiga toxin from *Shigella dysenteriae* (8).

Urinary tract infections in humans, especially the more severe form pyelonephritis, is predominantly caused by *E. coli*. Adhesion of the bacterium to the β-D-galabiose moieties of glycolipids present on urinary tract epithelial cells is considered to be an important virulence factor for development of the infection (9, 10, 11). Adhesion is mediated by P pili, which is the best characterized member of a family of hairlike extracellular protein appendages utilized by most gram-negative pathogens for specific binding to host ligands. The intricate mechanism and genetics of P pilus formation have been determined by Hultgren and Normark and their co-workers (12). Thus, P pili have the galabiose-binding adhesin, PapG, located at the end of the rodlike pilus, which is composed of a large number of PapA subunits and minor amounts of four other subunits. The molecular chaperone PapD is required for proper assembly of

this complex organelle. PapD forms bimolecular complexes with the pilus subunits in the bacterial periplasm and escorts them to the outer cell membrane, where they attach to the base of the growing pilus.

Streptococcus suis is an important gram-positive pathogen which causes meningitis, septicemia and pneumonia in pigs, and also meningitis in humans (13, 14). Many tissues in the pig express globotriosylceramide (Fig. 1) but a connection between the galabiose-specific adhesion of *S. suis* and pathogenesis still remains to be established, as do the details of adhesin presentation employed by the bacterium. In addition to the function as a ligand for bacteria, globotriosylceramide has been found to be a tumour associated antigen in Burkitt lymphoma (15), and glycolipids of the globoseries are also antigens on teratocarcinoma cells and have been suggested to be stage-specific developmental antigens (16).

We have investigated the recognition of cell surface carbohydrates by adhesin proteins from *E. coli* and *S. suis*. Extensive use has been made of deoxy- and deoxyfluoro derivatives of oligosaccharides to reveal the hydrogen bonding patterns between adhesins and carbohydrate ligands. Synthetic peptides have also been used to elucidate the details of binding between the protein subunits of the *E. coli* P pilus and the chaperone PapD. Both adhesin-receptor interactions and subunit-chaperone interactions constitute targets for design of drugs that impede the binding of pathogens to host cells.

The T cell immune response to derivatives of immunogenic peptides that carry carbohydrate haptens has also been studied using the tumour-associated antigen in Burkitt lymphoma as a model. Applications of an enhanced immune response against carbohydrate antigens may be useful in treatment of infectious disease and cancer.

BINDING OF BACTERIA TO SACCHARIDES OF THE GLOBOSERIES OF GLYCOLIPIDS

Mapping of the epitopes of saccharides that are recognized by proteins requires access to truncated oligomers corresponding to the natural receptor-active carbohydrates for initial identification of crucial monosaccharide units. A collection of deoxy- and deoxyfluoro derivatives of the minimum epitope (usually a disaccharide) can then be used for identifying hydroxyl groups involved in hydrogen bonding to the protein. This approach was pioneered by Lemieux and co-workers in their studies of the recognition of carbohydrates by antibodies and lectins (17).

We have synthesized a large number of derivatives of the globoseries of glycolipids for use in dissection of binding epitopes recognized by bacterial adhesins. More specifically, all di-, tri-, tetra-, and pentasaccharides corresponding to the Forssman antigen (Fig. 1) were prepared (18, 19), as well as all monodeoxy analogs and some deoxyfluoro analogs of lactose, galabiose and globotriose (20, 21, 22, 23, 24, 25, 26). The conformations of these analogs were calculated by various molecular mechanics methods (21, 22) and the minimum energy conformations were shown to be very similar to those of their natural counterparts (27, 28).

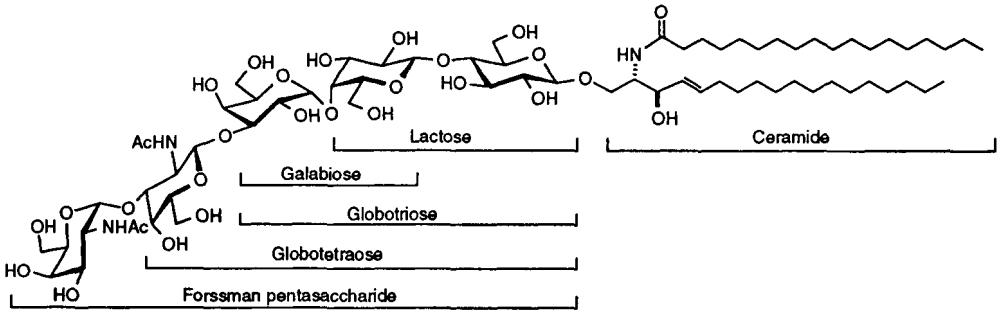

Fig. 1 Saccharides of the globoseries of glycolipids.

The saccharides were obtained as water soluble methyl or 2-(trimethylsilyl)ethyl (TMSEt) glycosides, suitable as inhibitors of carbohydrate-protein binding. In addition, the inherent synthetic

flexibility of the TMSEt glycosides (29, 30) permitted transformations into natural glycosyl ceramides (31) and artificial glycolipids (32), as well as spacer glycosides for coupling to proteins, particles, and surfaces (32). Examples of these compound types are discussed in a recent review on the synthesis of neoglycoconjugates (33).

The synthesis of unnatural deoxy- and deoxyfluoro sugars is considerably more demanding than their natural counterparts, which in many cases can be obtained by both organochemical and enzymatic methods. Deoxygenations of saccharides can be performed in good yields by the radical chemistry-based reduction procedures developed by Barton and co-workers (34) and deoxyfluorination is normally based on the use of reagents such as DAST (35) and TASF (36). As mentioned above, several complete sets of monodeoxy-oligosaccharides were synthesized via these methods.

When possible, standard methods of glycosylation were used in our synthetic work. However, as pointed out by Paulsen (37), every oligosaccharide synthesis is a unique endeavour and consequently, the synthesis of oligosaccharides is by no means a routine matter, especially when unnatural monosaccharide building blocks are to be integrated into the final structure.

The various synthetic analogs of the saccharides derived from the globoseries of glycolipids were used for mapping of binding epitopes of surface proteins in pathogenic *E. coli* and *S. suis* bacteria. In the case of the Gram negative *E. coli*, two different recombinant strains were used, carrying the sugar-binding pilus-associated adhesins $PapG_{J96}$ and $PapG_{AD110}$ (38). Both strains originated from infected humans, although the latter strain seems to be more closely correlated with human urinary tract infection (39).

Mapping of binding epitopes was based on either inhibition by soluble saccharides of the hemagglutination of human red blood cells (known to carry large amounts of the globoseries of glycolipids) by the bacteria under study (40, 41), or binding of bacteria to saccharides immobilized on microtiter plates (with or without inhibition by soluble saccharides) (42). The relative potency of the inhibitors in the hemagglutination assay was determined from the concentration that barely prohibited the formation of an agglutinate (IC_{50}), whereas in the microtiter plate assay, the amount of bound bacteria was determined by a standard ELISA technique employing anti-*E. coli* antibodies for quantification of bound bacteria.

The relative efficency of the truncated Forssman saccharides as inhibitors of hemagglutination or of adhesion to microtiter plates with *E. coli* is shown in Table 1 [absolute IC_{50} values can vary considerably between different batches of bacteria (41)]. The corresponding data for deoxy- and deoxyfluorogalabiosides is depicted in Fig. 2, including data for *S. suis* binding.

TABLE 1. Relative efficiency[a] of Forssman-related saccharides as inhibitors of binding of *E. coli* strains HB101/pPAP5 and HB101/pPIL110-35 that carry the $PapG_{J96}$ and $PapG_{AD110}$ adhesins, respectively.

	Relative inhibitory power (%)[a]		
Saccharide	Microtit. plate[b] pPAP5	Hemagglut. pPAP5	Hemagglut. pPIL110-35
GalNAcα3GalNAcβ3Galα4Galβ4GlcβOTMSEt[c]	18	n.d.	430
GalNAcβ3Galα4Galβ4GlcβOTMSEt	26	n.d.	560
Galα4Galβ4GlcβOTMSEt	11	8	420
Galβ4GlcβOEt	<2	<2	<30
GalNAcα3GalNAcβ3Galα4GalβOTMSEt	99	n.d.	170
GalNAcβ3Galα4GalβOTMSEt	55	n.d.	150
Galα4GalβOTMSEt	100	100	100
GalNAcα3GalNAcβ3GalαOMe	<2	n.d.	<30
GalNAcβ3GalαOMe	<2	n.d.	<30
GalNAcα3GalNAcβOMe	n.d.	n.d.	<30

[a] The inhibitory power of Galα4GalβOTMSEt was set to 100%. [b] Covalently coated with the globotetraoside GalNAcβ3Galα4Galβ4GlcβSCH$_2$CH$_2$COOH (42). [c] TMSEt = CH$_2$CH$_2$SiMe$_3$.

As seen in Table 1, the β-D-galabiose part (Galα4Galβ) of the saccharides is required in order to obtain efficient inhibition. Furthermore, strain HB101/pPAP5 prefers a hydrophobic aglycon on the Galβ unit over the glucose aglycon present in the naturally occurring globotriosides, whereas strain HB101/pPIL110-35 prefers the natural glucose extension (43). This raises the question about the nature of the natural receptor for the $PapG_{J96}$ adhesin in strain HB101/pPap5, since galabiosylceramide is a very rare glycolipid in human tissue.

The findings presented above are corroborated by the binding characteristics of various fluorescence-labeled *E. coli* strains to human kidney tissue, which expresses globotriosylceramide on the epithelial cell surfaces (39). In short, the HB101/pPAP5 strain does not bind, whereas the HB101/pPIL110-35 strain is an avid binder. A variant of the latter strain, having a knock-out mutation of the $PapG_{AD110}$ gene, did not bind. The same result was obtained with HB101/pPIL110-35 preincubated by TMSEt globotrioside (compound shown as entry 3 in Table 1).

The inhibitory efficiencies of the deoxy- and deoxyfluoro galabiosides (39, 40) is depicted in Fig. 2. Both *E. coli* strains (Fig. 2A and B) require the same five oxygens of the galabiose unit for efficient recognition, as demonstrated by the low relative inhibitory powers of the 6-, 2'-, 3'-, 4'-, and 6'-deoxy compounds. The 6-, 4'-, and 6'-deoxyfluoro compounds revealed the directionality of the saccharide-

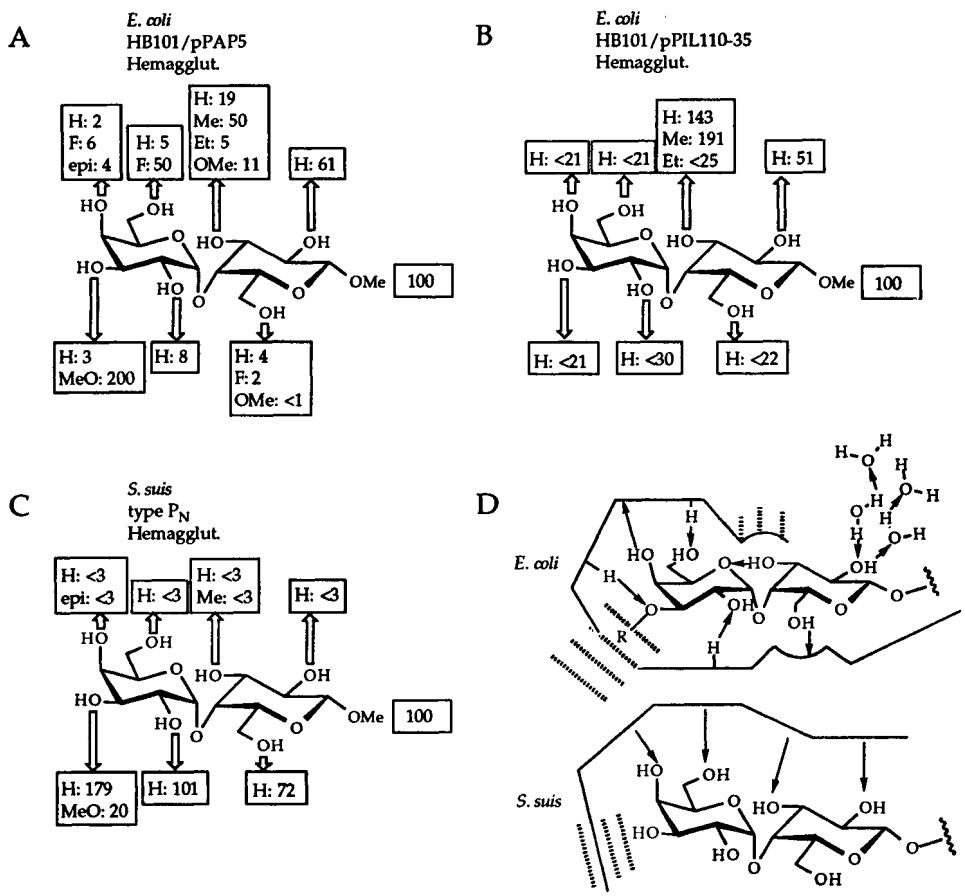

Fig. 2 **A-C**: Inhibition of hemagglutination of *E. coli* and *S. suis* strains by deoxy, deoxyfluoro, and other derivatives of methyl β-D-galabioside. The hydroxyl group substitution is shown in the squares together with the inhibitory power of the analog relative to that of the parent methyl galabioside, which was set to 100%. **D**: Receptor model for the binding of methyl β-D-galabioside to the *E. coli* and *S. suis* adhesins (arrows indicate intermolecular hydrogen bonds). For a detailed discussion, see references 39, 40, 44.

protein intermolecular hydrogen bonds. Finally, the efficiencies of the derivatives altered in the 3-position indicate the presence of a close hydrophobic fit between protein and saccharide, since the *C*-Me derivative is a good inhibitor whereas the *C*-Et derivative is too large to be accomodated in the protein receptor site. It is also noteworthy that the analog carrying a MeO group in the Galα moiety (Fig. 2A) is twice as efficient as the parent compound; the 3-position of Galα is glycosylated in the natural glycolipids, as shown in Fig. 1. The inhibition data presented in Fig. 2A and B leads to the saccharide—protein recognition model for *E. coli* shown in Fig. 2D. Both strains recognize roughly the same saccharide epitope despite the fact that there is only 46% sequence homology between the two PapG proteins (38).

Both *E. coli* and *S. suis* bacteria use the same natural galabiose-containing saccharides for binding, but the epitope recognized by *S. suis* (44) is quite different from that of *E. coli*. Only four hydroxyl groups (HO-2, -3, -4', and -6') are involved in hydrogen bonding, as shown in Fig. 2C. These groups are placed on one side of the galabiose molecule in contrast to the case for *E. coli*, which uses five hydroxyl groups placed on both sides of the molecule (Fig. 2D). The binding specificities described here constitutes the first example where two bacterial organisms of different origin recognize the same glycolipid, employing two different binding mechanisms.

The two sides of galabiosides are intersected by a rather large continuous hydrophobic surface made up of the ring hydrogens of the α-side of Galβ and the β-side of Galα. The arrangement with a continuous row of hydroxyl groups (or oxygens) close to a hydrophobic surface seems to be a common motif in protein-binding sugars, and several examples have been reported in saccharide-antibody recognition (17).

Mapping of the binding epitopes of receptor-active saccharides is a prerequisite for the design and synthesis of high-affinity analogs. The availability of such compounds would increase the likelihood of development of effective anti-adhesion-based drugs for treatment of bacterial infections. The increasing number of infections resistant against traditional antibiotics points towards the need for development of nontraditional therapeutic procedures. Inhibition of bacterial adhesion and/or interruption of the pilus-forming process in the bacteria (see below), might constitute useful mechanistic leads towards these goals.

STUDIES OF CHAPERONE SUBUNIT INTERACTIONS IN PILUS ASSEMBLY

PapD is regarded as the prototype bacterial periplasmic chaperone and has been found to have significant homology with more than 20 chaperones, which assemble pili in various pathogenic bacteria (45, 46). The proposed pilus subunit binding site in the cleft situated between the two domains of PapD was found to contain several amino acid residues that are conserved within the family of periplasmic chaperones, including the invariant Arg^8 and Lys^{112} (12). We found that formation of the complex between PapD and the adhesin PapG *in vivo* was abolished on deletion of the fourteen *C*-terminal residues in PapG, indicating that the *C*-terminus of the different pilus subunits are essential for binding to the PapD chaperone (47). In addition, the *C*-termini of pilus proteins show conserved features such as a pattern of alternating hydrophobic and hydrophilic residues, as well as a tyrosine/phenylalanine and a glycine at positions 2 and 14 from the *C*-terminus, respectively (48). Altogether these observations suggested that conserved features on the interactive surfaces of both periplasmic chaperones and pilus subunits are essential for chaperone-subunit complex formation and for proper pilus assembly.

To investigate the role of the conserved *C*-termini of the P pilus subunits in complex formation with the PapD chaperone we prepared (49) synthetic peptides, each of which corresponds to the 19 *C*-terminal residues in the subunits. In an enzyme-linked immunosorbent assay (ELISA) PapD bound well to the 19-residue peptide from the adhesin PapG (PapG296-314), and moderately or not at all to the *C*-terminal peptides from the other pilus subunits (48). The potent 19-mer PapG296-314 (Fig. 3A) was also found to inhibit PapD-mediated folding of denatured PapG, suggesting that the peptides indeed bound to the subunit binding site of PapD. This was further investigated using peptides as inhibitors of complex formation between PapD and the fusion protein MBP/G175-314 (50, 51). In MBP/G175-314, the *C*-terminal half of PapG which is responsible for binding to PapD, has been linked to the Maltose Binding

Protein (50). In this assay PapG296-314 and *N*-terminal truncates as short as the octamer PapG307-314, were found to inhibit complex formation between PapD and MBP/G175-314 (51). Thus, the octamer PapG307-314 was found to be the minimal peptide containing all functionalities necessary for binding to, and inhibition of PapD.

A

Gly^{296}-Lys-Arg-Lys-Pro-Gly-Glu-Leu-Ser-Gly-Ser-Met-Thr-Met-Val-Leu-Ser-Phe-Pro^{314}

B

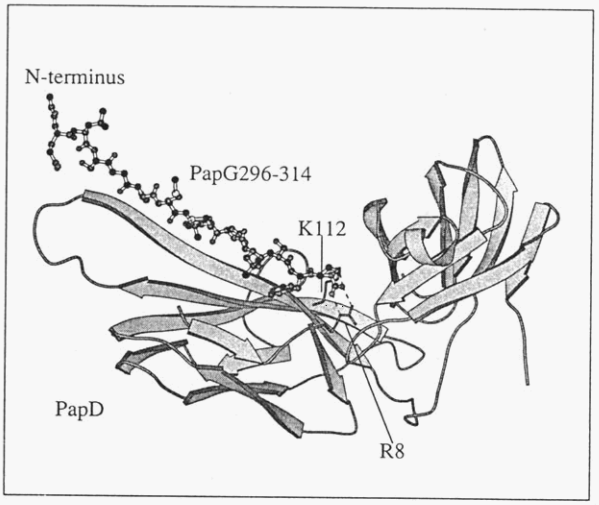

Fig. 3 **A.** Amino acid sequence of PapG296-314. **B.** Model of the structure of PapD co-crystallized with the PapG296-314 peptide, determined to 3.0 Å resolution (48). The peptide bound so as to extend a β-sheet in PapD and the carboxylate group of Pro^{314} was hydrogen bonded to residues Arg^{8} (R8) and Lys^{112} (K112) in PapD. (Courtesy of Dr. Derek J. Ogg, Garvan Institute of Medical Research, Sydney, Australia)

Further insight into the structural basis of the recognition of pilus subunits and related peptides by PapD was obtained from the crystal structure of PapD in complex with the peptide PapG296-314 at 3.0 Å resolution (Fig. 3B) (48). The peptide was bound in an extended conformation with the carboxylate group of the *C*-terminal Pro^{314} anchored by hydrogen bonds to the side chains of the invariant Arg^{8} and Lys^{112} in PapD. The peptide forms a parallel β-strand interaction with one of the β-sheets in PapD, mediated by seven main chain intermolecular hydrogen bonds. Interestingly, circular dichroism studies revealed a significant propensity for PapG296-314 to adopt a β-strand conformation in solution and a β-strand was also predicted as the preferred secondary structure for the peptide when integrated in PapG (49). Hydrogen bonding between PapD and PapG296-314 in the crystal involves only the *C*-terminal eight residues of the peptide in good agreement with the high inhibitory power of the octamer PapG307-314 discussed above. In the complex, the side chains of the peptide residues Met^{307}, Met^{309}, Leu^{311} and Phe^{313} make significant contacts with PapD. These residues are part of the conserved pattern of alternating hydrophobic and hydrophilic residues characteristic of the *C*-terminus of pilus subunits and their interactions with PapD most likely provides part of the explanation for the specificity of PapD for pilus related peptides and subunits (48). It can be expected that other parts of PapG also contribute to the interactive surface in the PapD-PapG complex and we recently showed that the peptide PapG175-190 represents one such epitope on PapG (50).

HELPER T CELL RESPONSE TO GLYCOPEPTIDES

Peptides play a crucial role in the immune response against protein antigens, *e.g.* from invading bacteria (52). The antigens are metabolized by macrophages and B cells (53) into peptides, usually composed of 14-

18 amino acids (54). The peptides are bound by MHC II molecules and the complex is presented on the cell surface for recognition by helper T cells. Recognition of such peptide-MHC II complexes stimulate clones of helper T cells which elicit a strong and lasting immune response directed towards the protein antigen. Protein antigens produced intracellularily, for example in cancer cells and virus infected cells, are handled in a similar way by MHC I molecules (52).

Carbohydrates are important antigens on cancer cells, infectious bacteria, and viruses, but they are not bound by MHC molecules (55, 56) and therefore give a weaker, T cell independent immune response. It is possible that conjugation of a carbohydrate antigen to a peptide presented by MHC molecules might enhance the immune response against carbohydrate antigens. We have therefore started an investigation of the helper T cell response towards *glyco*peptides (57, 58). As a model system we chose to couple the disaccharide β-D-galabiose, which is part of the tumour associated antigen in Burkitt Lymphoma (15), to a helper T cell stimulating peptide consisting of amino acids 52-61 from hen-egg lysozyme [HEL(52-61), Fig. 4] (59). Similar studies are also in progress in other laboratories (56, 60, 61).

To enable preparation of large numbers of glycosylated derivatives of HEL(52-61) for immunological studies we sought a simple and direct method for coupling of saccharides to spacers and amino acids. We found that mercaptopropionic acid, as well as hydroxyl and mercapto groups in the side chains of Fmoc-protected amino acids (Ser, Thr, Tyr, Hyp, Cys, homoCys), can be glycosylated without prior protection of their carboxyl groups (62, 63). When Lewis acids are used as promoters and carbohydrate 1,2-*trans* peracetates (Glc, Gal, Man, GlcNAc, cellobiose, lactose, galabiose) as glycosyl donors the desired glycosides were obtained in 34 to 65% yields. The building blocks had the protective groups of choice (64), i.e. *O*-acetyl and N^{α}-Fmoc, for direct use in solid phase glycopeptide synthesis. A conceivable drawback with the Fmoc-acetyl protective group combination in glycopeptide synthesis is the need for deprotection under basic conditions. This has caused great concern since carbohydrate chains linked to serine and threonine in glycoproteins can be removed by base-catalysed β-elimination, and amino acid residues can undergo base-catalysed epimerisation (65). However, we recently showed that neither Fmoc-deprotection under standard conditions with piperidine, nor deacetylation with sodium methoxide, hydrazine or ammonia, caused any β-elimination or epimerisation of peptide α-carbon stereocenters (66, 67).

HEL(52-61): Asp^{52}-Tyr^{53}-Gly-Ile-**Leu^{56}-Gln^{57}**-Ile-Asn-Ser^{60}-Arg

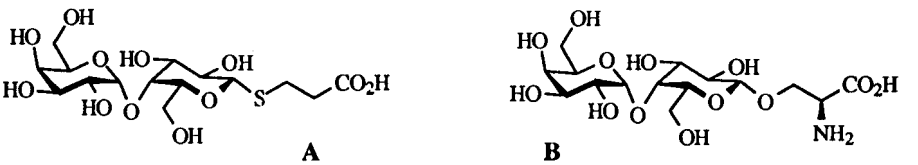

Fig. 4 Immunological studies were performed with derivatives of the T cell stimulating peptide HEL(52-61) in which, either β-D-galabiose attached to mercaptopropionic acid (**A**) was coupled to the *N*-terminus of the peptide, or β-D-galabiose attached to serine (**B**) was used to replace the residues shown in bold.

After coupling of β-D-galabiose octaacetate to mercaptopropionic acid and N^{α}-Fmoc serine, the five glycopeptide derivatives of HEL(52-61) indicated in Fig. 4 were prepared by solid phase synthesis (68, 69). Residues Tyr^{53}, Leu^{56}, and Gln^{57} have previously been shown to be important in contacting the T cell receptor (59) and were therefore chosen for replacement by β-D-galabiose linked to serine. The glycopeptides which carried β-D-galabiose at positions 57 and 60 were not bound by MHC II molecules and were therefore not immunogenic (Table 2) (58). The remaining three glycopeptides were bound well by I-A^{k} MHC II molecules and were used in immunizations of mice (57, 58). Helper T cell clones obtained after immunizations with the glycopeptides carrying the galabiose moiety at the α-amino group of Asp^{52} or at Ser^{53} were stimulated (measured as secreted IL-2) in an MHC restricted fashion by the respective glycopeptide immunogens but not by HEL(52-61). However, when the galabiose moiety in these two

glycopeptides was replaced by lactose or cellobiose T cell stimulation was retained, indicating that the specificity was directed towards a carbohydrate-induced conformation of the MHC-bound peptides (57, 58). In contrast, immunization with the glycopeptide that had β-D-galabiose linked to Ser^{56} elicited T cell clones that responded to the glycopeptide immunogen but not to HEL(52-61) or glycopeptide analogs in which the galabiose moiety was hepta-*O*-acetylated or replaced by cellobiose (58). This shows that carbohydrate moieties can indeed be part of the epitope recognized by helper T cells and we are now investigating the fine details of the T cell specificity. Applications of a carbohydrate specific response are found in attempts to elicit an enhanced immune response to glycoproteins that are part of foreign antigens such as bacteria, viruses or tumour cells.

TABLE 2. Immunological properties of neoglycopeptides obtained after attachment of galabiose at different positions in HEL(52-61)[a].

Immunological property	Modification in HEL(52-61)[b]: $Asp^{52}\rightarrow$ Gal_2-Mpa-Asp	$Tyr^{53}\rightarrow$ Gal_2-Ser	$Leu^{56}\rightarrow$ Gal_2-Ser	$Gln^{57}\rightarrow$ Gal_2-Ser	$Ser^{60}\rightarrow$ Gal_2-Ser
MHC-binding	+	+	+	-	-
Immunogenicity	+	+	+	-	n.d.
Carbohydrate specificity of T cells	-	-	+	n.d.	n.d.

[a] A plus (+) reveals that a glycopeptide displays the indicated immunological property, whereas a minus (-) indicates lack of this property. [b] Gal_2 = α-D-Gal*p*-(1→4)-β-D-Gal*p*, Mpa = 3-mercapto-propionic acid

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