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# TRANSFERENCE OF THE HIGH MOLECULAR WEIGHT CARBOHYDRATE SEQUENCES OF FETUIN TO THE SURFACE OF CARROT EMBRYO PROTOPLASTS

GRAHAM S. WARREN and MICHAEL W. FOWLER

*Wolfson Institute of Biotechnology, The University, Sheffield S10 2TN (U.K.)*

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**A method is described for the removal of the carbohydrate sequences of glycoproteins, and their covalent attachment to hydrocarbon chains. These synthetic membrane components may then be incorporated into liposome and cell membranes. Pronase-liberated glycopeptides derived from fetuin were linked by a reduced Schiff's base linkage to tetradecyl aldehyde. The resulting glycolipid was incorporated by external addition, into phosphatidylcholine liposomes. Glycolipid transfer to these liposomes rendered them suseptible to agglutination by wheat germ lectin, which binds *N*-acetylneuraminic acid, the terminal carbohydrate of the high molecular weight fetuin sugar sequence. Sequential removal of the terminal sugars, and subsequent agglutination behaviour towards various lectins, suggests that the carbohydrate sequence had been transfered intact. The glycolipid was incorporated into plant protoplast membranes by incubation with glycolipid-containing liposomes for 2 h at 37°C. These synthetic glycolipids may find a use in the study of carbohydrate-based recognition systems in animal and plant membranes. In addition they may prove useful in the development of cell and membrane tagging and handling techniques, by the insertion of sugar groups not normally present in these membranes.**

## Introduction

The central role of carbohydrate binding systems in many cell recognition phenomena is now well established [1,2]. Recognition can occur in the cytoplasm [3], at the membrane [4], or at the cell wall, if present [5]. The system can involve lectins or glycosyltransferases as the recognising molecule, and glycoproteins, glycolipids or structural carbohydrates as the recognised ligand [2].

The function of membrane-associated carbohydrate-recognition systems during animal development has been studied by direct addition to the cells of lectins which bind to, and thus block surface sugar residues [6]. Such blocking effects can result in specific structural abnormalities that can be correlated with the binding specificity of the lectin used. Using similar techniques, wall-associated recognition phenomena have been studied in higher plants [7], algae [8] and fungi [9]. However in cells possessing a wall, membrane associated mechanisms cannot be investigated in this way if the cell wall prevents access of molecules of the size of lectins to the membrane surface [10]. Furthermore, studies of wall-free protoplasts are difficult to interpret because intercellular organisation is destroyed and the membrane surfaces exposed may be artifacts of the isolation procedure.

The present work was undertaken in an attempt to develop an approach to the study of membrane-bound, carbohydrate-based recognition

Abbreviations; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; Neu*N*Ac, *N*-acetylneuraminic acid; Hepes, *N*-2-hydroxyethyl-piperazine-$N^1$-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid.

systems in plant cells. The technique consists of the synthesis of glycolipid molecules in which the sugar sequence can be precisely specified. When added to the membrane, the sugar sequences may act as non-functional analogues of endogenous effectors, i.e. they will block recognition, or alternatively they may prove to be functional recognition sequences and ellicit abnormal developmental effects.

Recently, glycolipids with a single defined sugar residue have been synthesised and inserted into liposome [11] and erythrocyte [12] membranes. The technique has not yet been extended to allow addition to the membrane of specified sugar sequences. We have chosen an alternative approach of transfer to the membrane of the carbohydrate sequences of glycoproteins, in the form of glycopeptides covalently linked to lipid anchor molecules. By this method it is possible to add a wide range of sugar sequences to membrane surfaces by modification of known glycoprotein chains [13] using specific glycosidases. This approach also allows evaluation of the role of endogenous glycoprotein carbohydrate sequences, as these can be isolated and subsequently re-inserted into membranes of cells at various developmental states.

The initial aim of the present work is to determine whether or not membrane-associated, carbohydrate-based recognition systems function in plant embryogenesis and meristem ontogeny. Plant membranes have been demonstrated to possess the components necesary for such systems, for example glycoproteins [14], lectins [15,16] and glycosyltransferases [17]. Although the cell wall provides a barrier to gross membrane contact between plant cells, it is possible that such communication can occur at localised areas of membrane contact between cells, for example at the plasmodesmata. The results presented in this paper represent the first stage in this programme, the coupling of a glycoprotein carbohydrate sequence to a lipid carrier and its functional incorporation into the plasma membrane. Part of this work has already been published in abstract form [18].

## Materials and Methods

*Isolation of glycopeptides*

150 mg of calf serum fetuin (Sigma, type 3) were digested with 5 mg of pronase (Sigma) in 10 ml of 50 mM Hepes buffer (pH 7.5) at 37°C. 0.1 ml of toluene was added to inhibit bacterial growth. After 24 h and 48 h, a further 3 mg of pronase and 0.1 ml of toluene were added and the pH was reset to 7.5. The digestion was terminated after 72 h as maximum cleavage of peptide bonds occurs in this period [19]. Insoluble material was removed from the digest by centrifugation for 5 min at 15000 × *g*. The supernatant was then fractionated on a 2.6 × 40 cm Sephadex G-50 (Pharmacia) column (Fig. 1). By comparison of these results with those of Spiro and Bhoyroo [19], the faster-eluting carbohydrate peak was taken to be a mixture of the high molecular weight glycopeptides of fetuin having the general structure [20]:

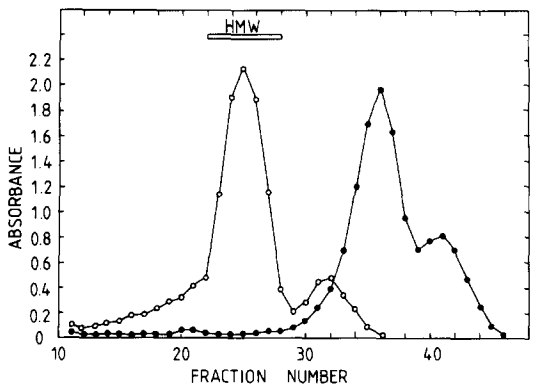
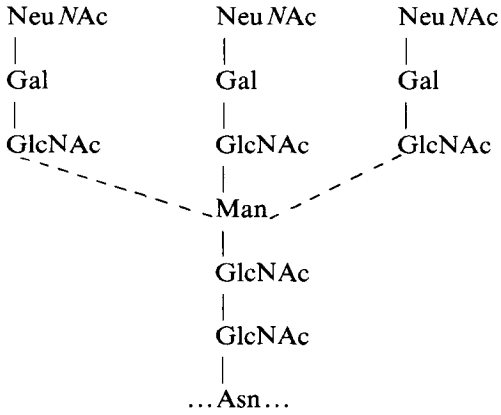



Fig. 1.Gel filtration of fetuin digest. Pronase-digested fetuin was fractionated on a 2.6×40 cm Sephadex G-50 column eluted with water at 0.5 ml·min$^{-1}$. 5-ml fractions were collected and assayed for carbohydrate (open circles) by the anthrone method [35]. Absorbance at 280 nm was taken as a measure of the amino acid and peptide concentration (closed circles). The fractions designated HMW were pooled and used for coupling to the aldehyde.



Similarly, the smaller carbohydrate peak represents the low molecular weight glycopeptides. The fractions designated HMW (Fig. 1) were pooled, lyophilised and redissolved in 1 ml of water for coupling, or 1 ml of agglutination medium (below) for agglutination assays.

*Coupling of glycopeptide to fatty aldehyde*

The glycopeptide was linked by its free amino group to the aldehyde function of tetradecyl aldehyde using sodium cyanoborohydride. This is a standard method of $NH_2$/CHO coupling [21]. A mixture containing 1 ml of glycopeptide solution (14 $\mu$mol -$NH_2$ as determined by the ninhydrin reaction [22]), 100 mg (0.47 mmol) of tetradecyl aldehyde (Aldrich) in 2 ml of chloroform, 10 ml of methanol and 200 mg (3.2 mmol) of sodium cyanoborohydride (Aldrich) was adjusted to pH 8.5 with NaOH and shaken for 24 h at 25°C. After this period, 10 ml of chloroform and 10 ml of water were added with vortexing, and the upper (methanol/water) layer collected. Insoluble material was removed by centrifugation for 5 min at 15000 × *g*, the supernatant was lyophilised and the residue dissolved in 2 ml methanol/water (1 : 1, v/v). The glycolipid was not further purified for this investigation.

The coupled mixture was characterised as follows. Samples of the glycolipid solution were run on 0.5 mm Silica gel H TLC plates developed in $CHCl_3$/MeOH/$NH_3$ (35%)/$H_2O$(160 : 20 : 7 : 3, v/v). Under these conditions the glycopeptide and coupled glycolipid remained at the origin and the tetradecyl aldehyde standard ran close to the solvent front. No tetradecyl aldehyde was detected in the coupled mixture (MeOH/$H_2O$ phase) as determined by iodine vapour staining (detection limit < 50 $\mu$g [23]).

The degree of coupling of aldehyde to glycopeptide was ascertained by determination of the number of free amino groups before and after the coupling procedure. As $NaCNBH_3$ was found to interfere with both the ninhydrin [22] and trinitrobenzene sulphonate [24] assays, the coupling mixture (MeOH/$H_2O$ phase) was fractionated, prior to assay, on a 30 × 1 cm Sephadex G-10 column eluted with MeOH/$H_2O$ (1 : 1, v/v) at 0.5 ml · $min^{-1}$. A clear separation of ninhydrin positive material from $NaCNBH_3$ or its breakdown products (determined by red colour with ninhydrin) was acheived. The recovery of glycopeptide from the column was 89%. By this procedure, the degree of coupling was determined as 85%.

*Addition of the glycolipid to liposomes*

40 mg of phosphatidylcholine (Sigma, type 1X-E) were dissolved in 1 ml of chloroform. The solution was evaporated to dryness in a stream of nitrogen and the residue was vortexed in 5 ml of an agglutination medium containing 0.5 M sorbitol, 0.2 M KCl, 1.4 mM $CaCl_2$, 0.9 mM $MnCl_2$ and 5 mM Hepes buffer (pH 7.5). The resulting suspension was centrifuged at 15000 × *g* for 5 min and the supernatant, containing multilamellar liposomes, was collected. To 2 ml of this liposome suspension was added 1 ml glycolipid solution and the mixture was vortexed. The suspension was standardised by dilution with agglutination medium to an absorbance value of 0.9 at 550 nm and at this density contained approx. 20 $\mu$g P per ml as determined by the Bartlett procedure [25], and (in washed liposomes) 0.05 $\mu$mol Neu*N*Ac per ml as assayed by the resorcinol method [26].

*Preparation of protoplasts*

Cell cultures were initiated from seedling roots of wild carrot (*Daucus carota* L.) by the method of Warren and Fowler [27]. Stock cell suspensions were subcultured every 14 days by transfer of cell clusters smaller than 250 $\mu$m, obtained by filtration through nylon mesh, to fresh medium at a dilution of approx. 4. Embryogenesis was initiated by subculture of the cells into medium lacking auxin, and protoplasts were obtained from these induced suspensions as follows. Approximately 1 ml packed volume of cells was incubated in 10 ml of a solution containing 2% (w/v) cellulase (Sigma), 0.5% (w/v) hemicellulase (Sigma), 0.5% (w/v) pectinase (Sigma), 0.7 M sorbitol and 5 mM Mes buffer (pH 5.6). The suspension was incubated at 25°C for 15 h on a rotatary shaker. Embryo protoplasts were smaller (15–20$\mu$m) than those derived from other cell types and were harvested by filtration through 20-$\mu$m nylon mesh. The protoplasts were then washed with, and resuspended in agglutination medium. Typically 80–90% of the protoplasts were viable as judged by the fluorescein diacetate method [28].

*Transfer of the glycolipid to protoplasts*

The glycolipid was added to protoplast membranes by exchange from liposomes as described by Rando et al. [12]. 1 ml of protoplast suspension in agglutination medium (approx. $10^6$ protoplasts) was added to 1 ml of the glycolipid-containing liposome suspension, and the mixture was incubated with gentle shaking for 2 h at 37°C. The protoplasts were harvested by centrifugation and washed with, and resuspended in agglutination medium. Protoplast viability was unimpaired by this procedure.

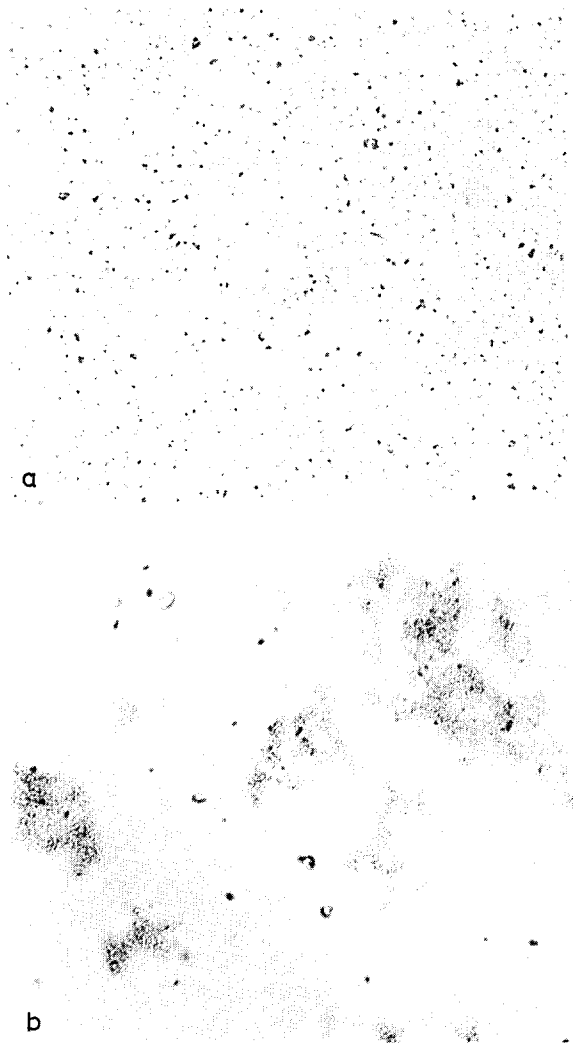



Fig. 2.Lectin-induced agglutination of liposomes. (a). Phosphatidylcholine liposomes exposed to 100 $\mu g \cdot ml^{-1}$ wheat germ lectin. Zero agglutination. (b). Glycolipid-containing liposomes agglutinated by 100 $\mu g \cdot ml^{-1}$ wheat germ lectin. Magnification $\times 100$.

*Sequential removal of the terminal region sugars*

The asialo-glycolipid was prepared exactly as above starting from desialised fetuin. The terminal Neu*N*Ac residues of fetuin were removed by heating 150 mg of the protein in 10 ml of 0.025 M $H_2SO_4$ at 80°C for 1 h [19].

The sub-terminal D-galactose residues were partially removed by treatment of 1 ml of liposome suspension (in agglutination medium) containing the glycolipid derived from desialised fetuin with 300 units of $\beta$-galactosidase (Sigma. ONPG units). The suspension was incubated at 37°C with gentle shaking for 3 h.

*Agglutination assay*

Agglutination assays were performed in watch glasses subjected to gentle rocking. The assay mixtures consisted of 0.05 ml of protoplast or liposome suspensions, and 0.05 ml of lectins and sugars as required. Wheat germ lectin and peanut lectin were obtained from Boehringer Ltd. and horse gram lectin from Sigma Ltd. Where appropriate the assay volumes were made up to the stated level with agglutination medium. After 10 min the watch glasses were examined under low magnification and the agglutination assessed. No significant evaporation occurred during the assay period.

In all the experiments reported agglutination was clearly present or absent. Agglutination of liposomes (Fig. 2) resulted in an increase in turbidity (absorbance at 550 nm) of about 30%. In the case of protoplasts, at least 75% of the cells agglutinated into clusters of 20 or more units.

## Results

The results presented in Table I show that treatment with the glycolipid rendered phosphatidylcholine liposomes susceptible to agglutination by wheat germ lectin. The effect is unlikely to be due to adsorption of the glycopeptide on to the membrane surface because coupling to the lipid anchor was required. Agglutination was abolished by GlcNAc but not by glucose, indicating that the binding was due to recognition of specific sugar

TABLE I

BEHAVIOUR OF GLYCOLIPID-CONTAINING LIPOSOMES TOWARDS VARIOUS LECTINS

Each assay contained a total volume of 0.15 ml and had a liposome density equivalent to an absorbance of 0.9 at 550 nm. Lectins were included at a concentration of 100 $\mu g \cdot ml^{-1}$. In cases where no agglutination occurred, the lectin concentration was increased in a second experiment to 500 $\mu g \cdot ml^{-1}$, this did not alter the experimental result in any instance. Sugars were present, where stated, at 2 $mg \cdot ml^{-1}$ and in every case lectin-mediated agglutination was inhibited by the appropriate sugars at this concentration. GL, glycolipid-containing; AGL, asialoglycolipid-containing; WGA, wheat germ lectin; PNA, peanut lectin; HGA, horse gram lectin.

| Treatment | Agglutination |
|---|---|
| PC liposomes | − |
| PC liposomes + Glycopeptide | − |
| PC liposomes + Glycopeptide + WGA | − |
| PC liposomes + WGA | − |
| PC liposomes + PNA | − |
| GL liposomes | − |
| GL liposomes + WGA | + |
| GL liposomes + PNA | − |
| GL liposomes + WGA + GlcNAc | − |
| GL liposomes + WGA + Glucose | + |
| AGL liposomes | − |
| AGL liposomes + WGA | − |
| AGL liposomes + PNA | + |
| AGL liposomes/β-galactosidase | − |
| AGL liposomes/β-galactosidase + WGA | + |
| AGL liposomes/β-galactosidase + PNA | + |
| AGL liposomes/β-galactosidase + HGA | − |

residues. Peters and co-workers [29] demonstrated that wheat germ lectin binds Neu*N*Ac at the same site as GlcNAc although with a much reduced affinity. Thus GlcNAc can competitively inhibit Neu*N*Ac binding to the lectin. Wheat germ lectin has been shown to possess a site capable of binding single sugars and short sugar sequences [30], and consequently the possibility arises that the lectin may recognise sugars intercalated in a carbohydrate sequence. The observation (Table I) that removal of Neu*N*Ac from the fetuin glycopeptide resulted in a loss of reactivity of the resulting glycolipid towards wheat germ lectin suggests that under our conditions, only the terminal Neu*N*Ac residue is bound by the lectin. We infer that the glycolipid had been incorporated into the liposome membranes with at least some of the sugar residues accessible from the outside. The lowest concentration of wheat germ lectin that was required for agglutination was 30 $\mu g \cdot ml^{-1}$.

The lack of agglutination of the glycolipid-containing liposomes by high levels of peanut lectin (which binds terminal galactose residues [31]), suggests that the terminal residues of the fetuin carbohydrate sequence were stable under the conditions of glycopeptide isolation and glycolipid synthesis. To provide further evidence that the fetuin sequence had been transferred intact, sugars were removed sequentially and the agglutination behaviour towards various lectins was studied (Table I). Liposomes containing the glycolipid prepared from desialised fetuin were not agglutinated by wheat germ lectin but were agglutinated by peanut lectin (terminal galactose present). Subsequent treatment of these liposomes with β-galactosidase allowed them to be agglutinated by wheat germ lectin (terminal Neu*N*Ac or GlcNAc present). The results from this series of experiments suggest the terminal sequence:

Neu*N*Ac-Gal-Neu*N*Ac/GlcNAc-

which corresponds to the published high molecular weight carbohydrate sequence for fetuin [20]. We infer that the sequence has been transferred without modification.

TABLE II

BEHAVIOUR OF GLYCOLIPID-CONTAINING PROTOPLASTS TOWARDS VARIOUS LECTINS

Each assay contained a total volume of 0.15 ml and a protoplast density of approx. $10^6$ $ml^{-1}$. Lectin and sugar concentrations were as described for Table I.

| Treatment | Agglutination |
|---|---|
| Protoplasts | − |
| Protoplasts + Glycopeptide | − |
| Protoplasts + Glycopeptide + WGA | − |
| Protoplasts + WGA | − |
| Protoplasts + PNA | + |
| GL protoplasts | − |
| GL protoplasts + WGA | + |
| GL protoplasts + WGA + GlcNAc | − |
| GL protoplasts + WGA + Glucose | + |
| AGL protoplasts + WGA | − |

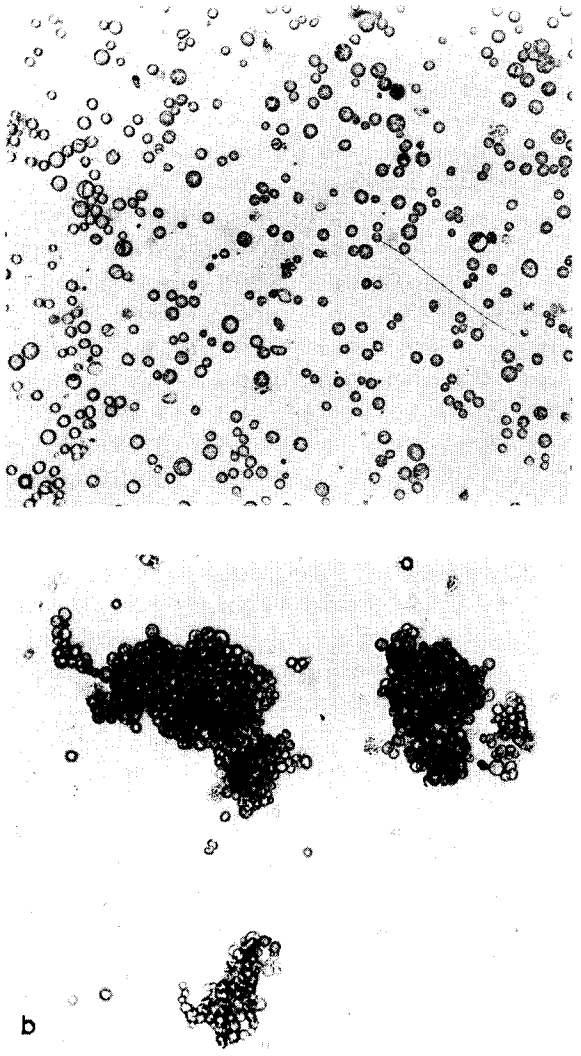



Fig. 3.Lectin-induced agglutination of protoplasts. (a). Carrot embryo protoplasts exposed to 100 $\mu g \cdot ml^{-1}$ wheat germ lectin. Zero agglutination. (b). Glycolipid-containing protoplasts agglutinated by 100 $\mu g \cdot ml^{-1}$ wheat germ lectin. Magnification $\times 100$.

The terminal sequence of Neu*N*Ac-Gal- is common to both the *N*-linked (high molecular weight) and *O*-linked (low molecualr weight) carbohydrate chains of fetuin. The third sugar in the sequence is GlcNAc in the high molecular weight chain and GalNAc in the low molecular weight chain [20]. Liposomes containing the desialised glycolipid treated with $\beta$-galactosidase were not agglutinated by high concentrations of horse gram lectin, which binds GalNAc, and thus there was little contamination of the high molecular weight fraction by the low molecular weight sequences (Table I).

Table II shows the results of experiments performed on carrot protoplasts containing the fetuin-derived glycolipid. Untreated protoplasts were not agglutinated by wheat germ lectin although they were susceptible to agglutination, for example by peanut lectin. Therefore carrot protoplasts do no carry accessible GlcNAc or Neu*N*Ac residues but do have surface galactose groups. Protoplasts incubated with glycolipid-containing liposomes for 2 h were strongly agglutinated by wheat germ lectin (Fig. 3b), and this observation indicates that the lipid had been transferred to the protoplasts membrane. The ability to be agglutinated by wheat germ lectin persisted for at least 3 h at 25°C after removal of the protoplasts from the liposome suspension. A similar experiment with the desialised glycolipid did not result in protoplast agglutination with this lectin (Table II).

Protoplasts containing the glycolipid remained viable, even when agglutinated, as judged by the fluorescein diacetate method [28].

## Discussion

The results suggest that synthetic glycolipids formed by coupling tetradecyl aldehyde to pronase-liberated glycopeptides can enter liposome and plant cell membranes as relatively stable membrane components. Transfer of the glycolipid to plant cell protoplasts allows the latter to be agglutinated by lectins, and this behaviour could provide the basis for cell and membrane tagging and handling techniques by the addition of sugar residues not normally present in these membranes. Recognition of added sugars by endogenous mechanisms provides the rationale for a technique for the study of cell communication phenomena as described above. If the glycolipids are to be of use in gaining information about the operation of carbohydrate-based recognition systems then they must satisfy several criteria, as described below.

Essentially they must have a relatively long lifetime in the membrane so that the cell surface is not depleted of funcional sites before the recognition events can occur. It is necessary therefore that

the glycolipids are not so hydrophilic that they are rapidly lost into solution. The observation that protoplast agglutination was possible for at least 3 h after glycolipid addition to the membranes suggests that such loss, if it occurs, is not particularly rapid in the present case. Similarly, the molecules must be metabolically stable, i.e. not rapidly degraded or modified by membrane or wall-located enzyme systems, and not readily internalised. In addition, in the case of walled cells, the glycolipids must be small enough to pass through the wall pores and reach the membrane surface. The physical stability of a molecule as a membrane component depends primarily upon its ratio of hydrophilic to hydrophobic residues. Thus for stability, a relatively long hydrophobic chain is required. However, lipid transfer to membranes appears to occur by monomer addition or exchange [12,32] and this process is facilitated for a relatively hydrophilic structure having a high critical micelle concentration. This constraint may be more severe in the case of glycolipid addition to plant cells in which the cell wall may hinder the passage of glycolipid molecules to the membrane surface. The optimal balance of hydrophilic and hydrophobic residues will have to be determined for each type of glycopeptide used and will depend on the number and type of amino acids retained in the glycopeptide, and the number and charge of the carbohydrates present. The hydrophobic nature of the glycolipid is essentially proportional to the length of the fatty aldehyde used in its synthesis and as such can be varied to meet the demands of a particular experiment. Before a critical comparison of the effects of a range of synthetic glycolipids can be made, it must be ascertained that the structural differences between individual glycolipid types do not result in changes in stability, turnover or ease of acess to the membrane.

On the basis of pulished data, the fetuin-derived glycolipid, in its non-associated form, should pass through the plant cell wall, and experiments are at present being carried out to confirm this. Hodgson et al. [33] found that polysaccharides of molecular weight greater than 1000 caused wilting in tomato seedlings. This toxicity was ascribed to a mechanical blocking effect, possibly of cell wall pores. The wilting effect increased linearly over the molecular weight range 1000–9000, indicating a decreasing permeability with increasing molecular weight. The fetuin-derived glycolipid has a molecular weight of approx. 4000 and so may have a restricted but still appreciable access to the membrane. It may prove necessary to use lower molecular weight carbohydrate sequences to obtain efficient transfer throught the cell wall.

As the lipid aldehyde is coupled to free amino groups in the glycopeptide, multiple coupling may occur if the glycopeptide contains basic amino acids. Such coupling could prove to be a problem if constraints were imposed on the orientation of the sugar sequence such that it was not easily accessible. However, the problem could be resolved by protection of free amino groups prior to glycopeptide isolation.

Williams and co-workers [34] have reported that synthetic glycolipids based on alkylamines caused lysis of erythrocytes, although Rando et al. [12] successfully incorporated a cholesterol-based monoglycolipid into similar cells. Thus before a carbohydrate-recognition system can be implicated, any effect that the glycolipids have on cell development must be shown to be dependent on the sugar sequence of the molecule and not due to a non-specific detergent action.

The directly-observed agglutination of glycolipid-containing liposomes as described in this paper, may itself prove to be a useful method for the investigation of lectin specificity towards sugar sequences as opposed to single sugars. In a similar way, the antigenic properties of the various carbohydrate sequences of glycoproteins could also be studied.

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