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Immunoglobulin superfamily members gp65 and gp55: tissue distribution of glycoforms

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Abstract Gp65 and gp55 are immunoglobulin superfamily members produced by alternative splicing of the same gene transcript, and originally identified as components of synaptic membranes. A monoclonal antibody specific for gp65 and gp55 has been used to detect immunoreactive species in a wide range of tissues. All immunoreactive species bind to concanavalin A and deglycosylation studies show that in all tissues tested other than brain the immunoreactive species are derived from gp55. HEK cells transfected with gp65 or gp55 express different glycoforms from brain showing that the pattern of glycosylation of these molecules is dependent upon the cell type in which they are expressed.

Key words: Immunoglobulin superfamily; Glycosylation; Glycoform; Synapse; Concanavalin A

1. Introduction

Gp65 and gp55 are two closely structurally related glycoproteins (apparent M_r 65 and 55 kDa respectively) originally identified as components of synaptic membranes (SM) [1,2]. Gp65 and gp55 are recognised by a single monoclonal antibody (mab), SMgp65. The two glycoproteins show striking differences in regional, subcellular and cellular distribution, and in developmental expression. Gp65 is most enriched in post-synaptic densities (PSDs) prepared from adult rat forebrain whereas gp55 is virtually absent from this fraction [1]. The PSD is a specialised region of the membrane cytoskeleton which both underlies and is attached to the post-synaptic membrane. Gp65 is localised to subsets of predominantly forebrain neurones whereas gp55 is present in all brain regions [1]. Gp55 is expressed early in brain development reaching its maximal level by postnatal days 10–12, whereas the level of gp65 increases markedly during the second and third postnatal weeks, concomitant with the major period of synaptogenesis [3]. Sequencing studies show that gp65 and gp55 are novel members of the immunoglobulin (Ig) superfamily which arise by alternative splicing of the same gene transcript. They contain a common sequence comprising two Ig domains, a single transmembrane domain and a short intracellular domain [4]. Gp65 is distinguished from gp55 by the presence of a further Ig domain at its N-terminus. The sequence data show that both gp65 and gp55 contain six potential sites for *N*-glycosylation. Enzymic deglycosylation with endoglycosidase H and endoglycosidase F (endo-F) containing peptide-N glycosidase F (PNGase F) shows that both glycoproteins contain high mannose and complex oligosaccharides. Complete removal of oligosaccharides with trifluoromethane sulphonic acid or endo-F/PNGase F lowers the apparent M_r of gp65 and gp55 to 40 and 28 kDa respectively [2]. These results suggest that several if not all of the six sites predicted from the sequence data are glycosylated in vivo.

Our original published data suggested that both glycoproteins are brain-specific [1]. However, Northern blot data shows that the 2.2 kb transcript which encodes gp55 is widely distributed, whereas the 2.5 kb transcript which encodes gp65 is brain-specific [4]. We have previously shown that gp55 glycoforms are present in liver [4]. Thus our aims here are: to demonstrate that although gp65 is brain-specific gp55 glycoforms are expressed in a wide range of tissues; to establish that the pattern of gp55 glycoforms varies between tissues, and, using gp65 and gp55 transfected HEK cells, to demonstrate that the pattern of glycosylation of both molecules is dependent upon the cell type in which they are expressed.

2. Materials and methods

2.1. Preparation of Triton X-100 solubilised membrane proteins

Triton X-100 solubilised membrane proteins from all tissues tested were prepared from adult Wistar rats (postnatal age 13–15 weeks) as follows. Immediately following removal from the animal all tissues were homogenised in homogenising buffer: 25 mM Tris-HCl, pH 7.4, 500 mM NaCl containing the following protease inhibitors: antipain, chymostatin, leupeptin and pepstatin all at a final concentration of 1 mg/ml and phenylmethylsulphonyl fluoride (0.2 mM). The tissue to buffer ratio was 5 ml buffer/g wet wt. tissue. The homogenate (protein concentration 12–15 mg/ml buffer) was centrifuged at $100\,000 \times g_{av}$ for 1 h, and the pellet resuspended in an equal volume of homogenisation buffer containing 1% (v/v) Triton X-100. Following incubation for 60 min at 4°C the material was centrifuged as above. The supernatant contained the Triton X-100 solubilised proteins (protein concentration 4–5 mg/ml buffer). Thus about one third of starting protein is present in the Triton X-100 solubilised fraction. Protein samples were stored at −70°C prior to further analysis.

2.2. Isolation of concanavalin A (Con A) binding glycoproteins

High mannose containing glycoproteins were isolated by binding to Con A agarose beads as detailed by Gurd et al. [5]. The bound glycoproteins were eluted from the beads by boiling in Laemmli sample buffer for 5 min.

2.3. Digestion with endo-F/PNGase F

Digestion was performed as described by the manufacturer of the enzyme (Oxford GlycoSystems, Abingdon, UK). Membrane samples (200 μg) were denatured by boiling for 3 min in 0.5% (w/v) SDS containing 2.5% β-mercaptoethanol followed by digestion with endo-F/PNGase F (10 DGU/200 μg protein) in incubation buffer (20 mM sodium phosphate pH 7.5, 50 mM EDTA, 0.5% octylglyco-

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Abbreviations: Con A, concanavalin A; endo-F/PNGase F, endoglycosidase F/peptide-N glycosidase F; Ig, immunoglobulin; mab, monoclonal antibody; PBS, phosphate-buffered saline; PSD, post-synaptic density; SM, synaptic membrane

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side, 0.1% SDS, 0.5% β-mercaptoethanol) at 37°C for 18 h. Control samples were digested in the absence of the enzyme mixture. Protease inhibitors were included in all samples.

2.4. Gel electrophoresis and Western blotting

These were carried out as previously described ([2,6] and [7]). Proteins were separated on 10% gels unless otherwise stated.

2.5. Immunodevelopment of Western blots

This was essentially as described by Willmott et al. [2]. Briefly blots were blocked in 5% (w/v) dried milk dissolved in phosphate-buffered saline (PBS) and incubated in mab SMgp65 overnight. After washing four times in PBS containing 0.1% (v/v) Tween blots were incubated in peroxidase-coupled rabbit anti-mouse IgG (DAKO) and visualised using the ECL detection system.

2.6. Antibody production

Mab SMgp65 is secreted by a hybridoma cell line raised against a synaptic membrane Con A binding fraction [1]. The antiserum specific for gp65 was obtained by affinity purification of a rabbit polyclonal antiserum raised against a recombinant protein encoding all three gp65 Ig domains. Briefly fusion protein encoding the gp65 specific domain was separated by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. The nitrocellulose containing the bound gp65-specific domain was blocked with 5% (v/v) dried milk in PBS and incubated overnight in antiserum. The nitrocellulose was washed as for Western blots and gp65-specific antiserum eluted by incubation (5 min) in 0.2 M glycine, pH 2.8, containing 0.5 M NaCl. The small quantity of eluted gp65-specific antiserum was rapidly neutralised with saturated Tris.

2.7. Stable expression of gp65 and gp55 in HEK293 cells

This was as previously described [4].

3. Results

Immunodevelopment of a Western blot of Triton X-100 solubilised membrane proteins prepared from a range of tissues with mab SMgp65 shows the presence of immunoreactive species in all samples (Fig. 1). Tissues examined were liver, kidney, muscle, stomach, spleen, testis, lung, heart and brain (Fig. 1, lanes 1–9). None of the immunoreactive species detected with mab SMgp65 were observed on blots developed in the absence of primary antibody thus confirming the specificity of the antibody (results not shown). The diffuse nature of the immunoreactive bands is attributable to microheterogeneity of oligosaccharide structure as we have previously discussed [1,2]. As expected brain shows the two immunoreactive bands of apparent M_r 55 kDa and 65 kDa corresponding to gp65 and gp55 (Fig. 1, lane 9). Although the immunoreactive species detected in the other tissues are in the same molecular weight range none are of identical M_r to gp65 and gp55. All tissues tested contain an immunoreactive band of M_r 44 kDa although this is a very minor immunoreactive component in brain (Fig. 1, lower arrowheads). They also contain at least one further immunoreactive species, in some cases (e.g. heart) more, but the apparent M_r varies between tissues. Possible overlap between diffuse bands makes it difficult to unequivocally identify the exact number of glycoforms present in each tissue. However, the number of gp55 glycoforms does vary between tissues, one major species being detected in brain and at least four species in heart (Fig. 1, cf. lanes 9 and 8 respectively). Liver and muscle contain an immunoreactive species of apparent M_r 61 kDa, i.e. intermediate between that of gp65 and gp55 (Fig. 1, upper arrowhead lane 1). Lung contains a second prominent immunoreactive species, but in this case it is a diffuse band of apparent M_r 63 kDa (Fig. 1, arrowheads lane 7), which is distinct from both gp65

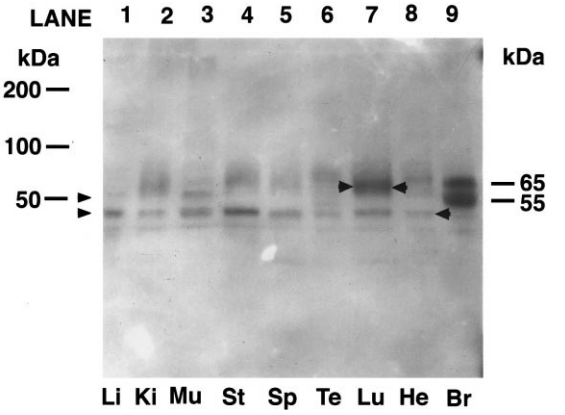

Fig. 1. An antibody specific for gp65 and gp55, mab SMgp65, recognises immunoreactive species in a range of tissues. A Western blot of samples of solubilised membrane proteins prepared from a range of tissues was immunodeveloped with mab SMgp65. The samples in order of loading from lane 1 to 9 were prepared from liver (Li), kidney (Ki), skeletal muscle (Mu), stomach (St), spleen (Sp), testis (Te), lung (Lu), heart (He) and brain (Br). Lower arrowheads indicate an immunoreactive band of apparent M_r 44 kDa. Upper arrowhead lane 1 and arrowheads lane 7 indicate immunoreactive bands of apparent M_r 61 and 63 kDa respectively. Protein loading: 20 μg/track.

and the 61 kDa species detected in liver. Diffuse higher M_r species are detected in other tissues such as stomach and testis (Fig. 1, lanes 4 and 6).

In order to confirm that, as for gp65 and gp55 from brain, the immunoreactive species detected in other tissues contain high mannose oligosaccharide residues, Con A binding experiments were carried out using solubilised membrane proteins from liver, muscle, lung and brain. Liver and muscle show patterns of immunoreactivity common to several tissues, while the lung and brain patterns are unique among the tissues tested. A Western blot of samples of the starting material and the eluted Con A binding glycoproteins is shown in Fig. 2. The results show that the 61 and 44 kDa immunoreactive species detected in liver and muscle are enriched in the Con A binding material (Fig. 2, lanes 2 and 4 respectively) compared to the starting material (Fig. 2, lanes 1 and 3

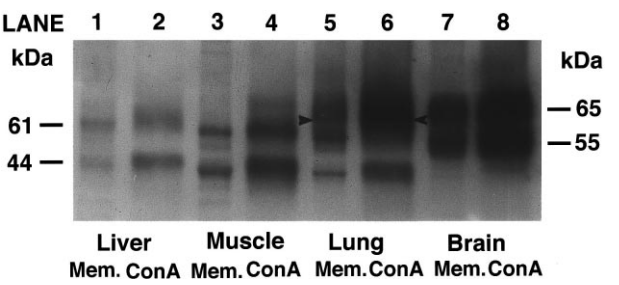

Fig. 2. Immunoreactive species recognised by mab SMgp65 in liver, muscle, lung and brain bind to Con A. A Western blot of detergent solubilised membrane protein (Mem.) and Con A binding material (Con A) from liver, muscle, lung and brain was immunodeveloped with mab SMgp65. Odd and even lanes represent solubilised membrane protein and Con A binding material from the same tissue. Thus lanes 1, 3, 5 and 7 are solubilised membrane protein and lanes 2, 4, 6 and 8 are Con A binding material from liver, muscle, lung and brain. Arrowheads lanes 5 and 6 denote a 63 kDa immunoreactive species. Protein loading was 20 μg/lane for membrane proteins. The loading of Con A binding material represented the amount extracted from 40 μg of membrane protein.

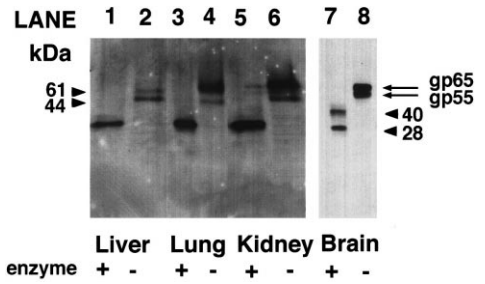

Fig. 3. Deglycosylation of immunoreactive species recognised by mab SMgp65 in liver, kidney, lung and brain with endo-F/PNGase F. Western blot of samples of solubilised membrane proteins from liver, lung, kidney and brain incubated in the presence or absence of endo-F/PNGase F. Control and endo-F/PNGase F digested samples from the same tissue are in adjacent lanes. Thus lanes 2, 4, 6 and 8 are control digested samples and lanes 1, 3, 5 and 7 are endo-F/PNGase F digested samples from liver, lung, kidney and brain. Enzyme indicates the combination of endo-F/PNGase F. Protein loading: liver and kidney 40 μg/track, lung 15 μg/track, brain 5 μg/track.

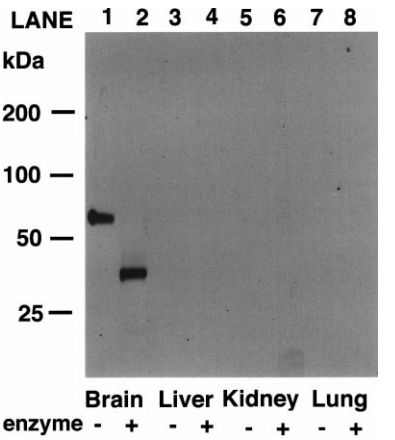

Fig. 4. An antibody specific for gp65 does not recognise immunoreactive species in liver, kidney and lung. A Western blot of control and endo-F/PNGase F digested samples immunodeveloped with antiserum specific for gp65 prepared as described in Section 2. Lanes 1, 3, 5 and 7 are control and lanes 2, 4, 6 and 8 are endo-F/PNGase F digested samples from brain, liver, kidney and lung. Gp65 immunoreactivity is detected only in control and endo-F/PNGase F digested samples from brain. Protein loading: 20 μg/track.

respectively) as is the 63 kDa species present in lung (Fig. 2, arrowheads lanes 5 and 6 respectively). As expected the brain Con A binding fraction is enriched in gp65 and gp55 compared to the starting material (Fig. 2, lanes 7 and 8 respectively). The apparent increase in the M_r of the immunoreactive glycoprotein species in the Con A binding compared to the starting material is due to the large amounts of Con A present in the samples which interferes with electrophoretic migration. The Con A is shed by the agarose beads during solubilisation of the binding proteins for SDS polyacrylamide gel electrophoresis.

In order to provide evidence that the immunoreactive species detected in tissues other than brain arise from gp55 samples of membrane proteins prepared from liver, kidney, lung and brain were enzymically deglycosylated using endo-F/PNGase F. This enzyme combination removes all *N*-linked oligosaccharides from glycoproteins. The results for brain show prominent bands of M_r 40 and 28 kDa in the deglycosylated compared to the non-deglycosylated sample (Fig. 3, lanes 7 and 8 respectively). These correspond to the previously established M_r values for deglycosylated gp65 and gp55 from brain [2]. The endo-F/PNGase F treated samples from liver, lung and kidney (lanes 1, 3 and 5 respectively) all show prominent deglycosylated bands of M_r 28 kDa, i.e. identical to the M_r of deglycosylated gp55, but no 40 kDa gp65 derived immunoreactive product.

Our data lead us to conclude that the novel immunoreactive species detected by mab SMgp65 in tissues other than brain are derived from gp55, but not gp65. This is confirmed by immunodevelopment of a Western blot of control and digested samples of brain, liver, kidney and lung with affinity purified gp65-specific antiserum (Fig. 4). As expected the antiserum recognises gp65 and its deglycosylated 40 kDa product in the control digested (Fig. 4, lane 1) and endo-F/PNGase F treated (Fig. 4, lane 2) samples of brain. No immunoreactive bands were detected in the other tissues. As the sequence of gp55 is common to both glycoproteins it is not possible to repeat the experiment with an antibody specific for this molecule.

The detection of at least two (and in some cases more) immunoreactive bands derived from gp55 in many of the tissues examined raises the possibility that the lower M_r species artefactually arises from the higher due to the action of endogenous glycosidases during sample preparation. In order to rapidly denature such enzymes and so discount this possibility samples of adult brain, liver, kidney and lung were immersed in boiling buffer for 5 min immediately following removal and prior to homogenisation. The results (not shown) confirm the same pattern of immunoreactive bands are present in both boiled and control samples.

The present results suggest that the pattern of gp55 glycosylation is dependent upon the cell type expressing the glycoprotein. To further investigate this and test if the same would be true for gp65, the glycoforms present in HEK293 cells stably transfected with cDNAs encoding either gp65 or gp55

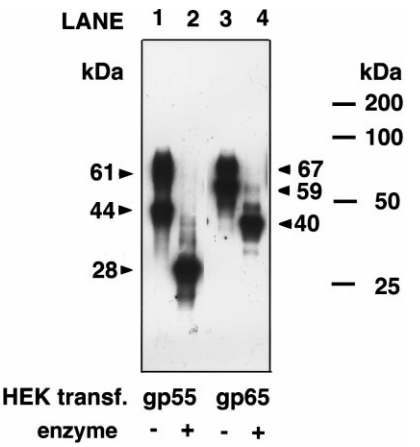

Fig. 5. Glycoforms detected in gp65 and gp55 transfected HEK cells differ from those detected in brain. A Western blot of control and endo-F/PNGase F digested samples of HEK cells transfected with gp65 or gp55 immunodeveloped with mab SMgp65. Lanes 1 and 2 show control and endo-F/PNGase F digested samples of gp55 transfected cells. Lanes 3 and 4 show control and endo-F/PNGase F digested samples of gp65 transfected cells. Protein loading: 20 μg/track.

were investigated. We have previously shown that control HEK293 cells are not immunoreactive. The pattern of immunoreactive species recognised by mab SMgp65 in control and deglycosylated samples of the gp65 and gp55 transfected cells is shown in Fig. 5. Two major bands of M_r 61 and 44 kDa were detected in the gp55 transfected cells (Fig. 5, lane 1). As expected a single 28 kDa immunoreactive species only was observed following endo-F/PNGase F digestion (Fig. 5, lane 2). The higher molecular mass minor immunoreactive components almost certainly represent low levels of partially deglycosylated intermediates as we have previously demonstrated for brain [2]. The gp65 transfected cells also expressed two immunoreactive species, but in this case of apparent M_r 67 and 59 kDa (Fig. 5, lane 3) which yielded the expected 40 kDa peptide product following endo-F/PNGase F treatment (Fig. 5, lane 4). Thus the pattern of glycosylation of both of these Ig superfamily members is clearly dependent upon the cell type in which they are expressed.

4. Discussion

The current results demonstrate that unlike gp65, gp55 glycoforms are expressed in a wide range of tissues. A number of these, notably the 55 kDa brain glycoform and the 63 kDa species detected in lung, have a restricted tissue distribution and may indeed be tissue specific. Other glycoforms are much more widely distributed, notably the 44 kDa species which was detected in all tissues examined. Variation in glycoforms is well established for many glycoproteins (for reviews see [8,9]). Perhaps the best characterised example is the Thy-1 molecule which is expressed in brain and thymus. This smallest member of the Ig superfamily is *N*-glycosylated at three sites giving rise to a number of glycoforms [8]. Strikingly there is no overlap between brain and thymus glycoforms.

Significantly tissue specific glycosylation has been reported for the chick blood-brain barrier glycoprotein neurothelin/(HT7/5A11 antigen) and its mouse homologue, basigin [10,11]. These molecules are the Ig superfamily members which share closest sequence homology (40–44%) with gp55. In retina 5A11/HT7 glycoforms with M_rs of 69 and 45.5 kDa are observed [10]. Immunoreactive bands of M_r 46–50 kDa are detected in erythrocytes, liver and kidney. The M_r of the 45.5–50 kDa molecular species from all tissue was lowered to 32 kDa following enzymic deglycosylation confirming that the differences in molecular mass are associated with differences in glycosylation. The apparent M_r of the 69 kDa species from retina was lowered to only 46 kDa following deglycosylation. This raises the possibility that the 69 kDa species, like gp65, has three Ig domains rather than the two Ig domains described for the lower M_r 5A11/HT7 species. Indeed the corresponding exon is contained in the basigin gene [4,12]. Fadool and Linser [10] attributed much of the observed difference in M_r between 5A11/HT7 glycoforms to differences in sialic acid content. However, the apparent M_rs of gp65 and gp55 from brain are lowered by only 2–3 kDa following digestion with neuraminidase [2].

The pattern of glycosylation of gp65 and gp55 is at least in part determined by the cell type in which they are expressed. This is best illustrated by the HEK293 cells transfected with cDNAs encoding gp55 and gp65. The gp55 glycoforms expressed in the transfected cells are of identical M_r to those found in several other tissues including kidney and liver. Strikingly two gp65 glycoforms of M_r 67 and 59 kDa are expressed in the transfected cells, compared to the single diffuse 65 kDa species observed in brain. This plausibly suggests that both gp65 and gp55 may have a similar pattern of glycosylation in the transfected cells which is distinct from that of brain. The patterns of glycosylation in brain do show specific trends which differ from other tissues. In particular there is a dominance of anionic oligosaccharides and by complex glycans, often fucosylated [9]. The brain glycoforms of gp65 and gp55 have a similar gross carbohydrate composition, although 2-D gel electrophoresis shows differences in microheterogeneity between gp65 and gp55 consistent with some differences in precise oligosaccharide structure. In this context gp65 contains no additional *N*-glycosylation sites to gp55.

Variation in the oligosaccharide structure of glycoproteins has been shown to have important functional consequences, for example, in regulation of cell surface events such as recognition and adhesion phenomena [13,14]. Thus homophilic adhesion between NCAMs is regulated in part by the content of polysialic acid chains [15,16]. *Cis* interaction between NCAM and L1 is inhibited by castanospermine, an alkaloid which interferes with processing of high mannose to complex and hybrid oligosaccharides [17]. The HNK-1 sulphated glucuronic acid glycan is implicated in cell-substrate adhesion via interactions with the G2 domain of laminin α chain [13,18]. Modification of the terminal sugar residues of oligosaccharides present on glycoproteins by surface galactosyl transferases is suggested to alter neurite outgrowth [19]. Changes in fucosylation have been implicated in learning in chicks [20] and in long-term potentiation in the rat [21]. Thus tissue variation in the oligosaccharide composition of gp55 may modulate its function.

The functions of gp65 and gp55 are not yet known. The available evidence suggests a role as positive or negative effectors of cell recognition and adhesion is most likely. The localisation of gp65 to the processes and terminals of subsets of neurones suggests that it may have an adhesive function at these loci whereas the widespread distribution of gp55 is consistent with an adhesive role between a wide range of cell types. Experiments designed to investigate the functions of these molecules will have to take into account the possible effect of variations in glycosylation.

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References

[1] Hill, I., Selkirk, C.P., Hawkes, R.B. and Beesley, P.W. (1988) Brain Res. 461, 27–43.
[2] Willmott, T., Skitsa, I., Hill, I., Mummery, R. and Beesley, P.W. (1992) J. Neurochem. 58, 2037–2043.
[3] Hill, I., Willmott, T., Skitsa, I., Selkirk, C., Murphy, S., Gordon-Weeks, P. and Beesley, P.W. (1989) Biochem. Soc. Trans. 17, 770–771.
[4] Langnaese, K., Beesley, P.W. and Gundelfinger, E. (1997) J. Biol. Chem. 272, 821–827.
[5] Gurd, J.W., Bisson, N. and Soulliere, J. (1992) Neuroprotocols 1, 207–214.
[6] Laemmli, U.K. (1970) Nature 227, 680–685.
[7] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.

[8] Rademacher, T.W., Parekh, R.B. and Dwek, R.A. (1988) Annu. Rev. Biochem. 57, 785–838.
[9] Wing, D.R. (1994) in: Glycobiology and the Brain (Nicolin, M. and Zatta, P.F., Eds.), Pergamon Press, Oxford.
[10] Fadool, J.M. and Linser, P.J. (1993) J. Neurochem. 60, 1354–1364.
[11] Kanekura, T., Miyauci, T., Tashiro, M. and Muramatsu, T. (1991) Cell Struct. Funct. 16, 23–30.
[12] Miyauchi, T., Jimma, F., Igakura, T., Yu, S., Ozawa, M. and Muramatsu, T. (1995) J. Biochem. (Tokyo) 118, 717–724.
[13] Schachner, M. and Martini, R. (1995) Trends Neurosci. 18, 183–191.
[14] Krog, L. and Bock, E. (1992) in: Carbohydrate Pathology (Dabelstein, E. and Clausen, H., Eds.), Munksgaard, Copenhagen.
[15] Rutishauser, U., Watanabe, M., Silver, J., Troy, F.A. and Vimr, E.R. (1985) J. Cell Biol. 101, 1842–1849.
[16] Moran, N. and Bock, E. (1988) FEBS Lett. 242, 121–124.
[17] Kadmon, G., Kowitz, A., Altevogt, P. and Schachner, M. (1990) J. Cell Biol. 110, 209–218.
[18] Hall, H., Vorherr, T. and Schachner, M. (1995) Glycobiology 4, 435–441.
[19] Begovac, P.C. and Shur, B.D. (1990) J. Cell Biol. 110, 461–470.
[20] Sukumar, R., Rose, S.P.R. and Burgoyne, R.D. (1980) J. Neurochem. 34, 1000–1006.
[21] Angenstein, F., Matthies, H., Staeck, S., Reymann, K.G. and Staak, S. (1992) Neurochem. Int. 21, 403–408.