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Glycosylation of the Alpha and Beta Tubulin by Sialyloligosaccharides

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ABSTRACT—To examine whether α and β tubulin are glycoproteins, we used a pyridylamino labeling method and a monoclonal antibody, SG3-1, raised against NeuAcα2-3Gal structure. Alpha and β tubulin from both pig brain and HeLa cells were positive for the SG3-1 antibody by immunoblot assay. Sialidase treatment reduced the reactivity of the SG3-1 antibody to α and β tubulin molecules. *N*-linked oligosaccharide analysis also showed that α and β tubulin are glycosylated. Moreover, immunofluorescence analysis showed that the filamentous structure recognized by the SG3-1 antibody was overlapped with microtubules, especially in the vicinity of the nucleus. These results indicate that α and β tubulin are glycosylated with sialyloligosaccharides.

Key words: glycosylation, monoclonal antibody, post-translational modification, sialic acid, tubulin

INTRODUCTION

Microtubules (MTs) are major cytoskeletal structures that play critical roles in a large number of cellular events including mitosis, ciliary and flagellar motility, intracellular transport and defining of cell morphology and polarity (Hyams and Lloyd, 1993). Alpha and β tubulin, the major components of MTs, are highly heterogeneous molecules (Field *et al*., 1984). This molecular heterogeneity results partly from multigene families (Luduena, 1998) and/or partly from a variety of post-translational modifications including phosphorylation (Eipper, 1972), tyrosination/detyrosination (Argarana *et al*., 1977), acetylation/deacetylation (L'Hernault and Rosenbaum, 1985), polyglutamylation (Edde *et al*., 1990), glycation (Cullum *et al*., 1991), the generation of non-tyrosinable α tubulin (Paturle-Lafanechere *et al*., 1991), polyglycylation (Redeker *et al*., 1994), palmitoylation (Caron, 1997), and glycosylation (Margolis *et al*., 1972). It is thought that this heterogeneity of tubulin molecules contributes to the diversity of MTs functions, however, the roles of each gene of the products and post-translational modifications are poorly understood (Rosenbaum, 2000).

There are several reports on tubulin glycosylation although it is generally accepted that cytosolic proteins are not glycosylated except for the *O*-GlcNAcylation. Metabolic labeling experiments showed that α and β tubulin are labeled with [^{14}C]-glucosamine in the mouse brain (Feit and Shelanski, 1975). Electron microscopic investigations also indicate that Concanavalin A reacts with MTs both *in vitro* (Huttich *et al*., 1977) and *in vivo* (Behnke, 1975; Prus and Mattisson, 1979). Biochemical analyses showed that tubulin contains carbohydrate, but amount of the carbohydrate varies with individual reports (Eipper, 1972; Margolis *et al*., 1972; Prus and Mattisson, 1979).

Since the 1980s, remarkable progress has been made in the detection methods for glycoconjugates using monoclonal antibodies (Hakomori, 1984) and pyridylamino (PA-) labeling (Hase, 1993). These methods are also useful to examine the features of α and β tubulin. In this study, we used monoclonal antibody SG3-1, which specifically recognizes the NeuAcα2-3Gal structure, and the PA-labeling method and obtained evidence indicating that sialyloligosaccharides are bound to α and β tubulin.

MATERIALS AND METHODS

Materials

Glycolipids such as NeuAcα2-3Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-1'Cer (GM1b), NeuAcα2-3Galβ1-4Glcβ1-1'Cer (GM3), NeuAcα2-3Galb1-1'Cer (GM4), NeuAcα2-1'Cer (GM5), NeuAcα2-6Galβ1-4Glcβ1-1'Cer (2-6GM3), NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1'Cer (GD3), NeuAcα2-3Galβ1-4(Fucβ1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer

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(sialyl Le^X), Galβ1-1'Cer (CMH) and Galβ1-4Glcβ1-1'Cer (CDH) were chemically synthesized (Kawamura *et al*., 1990). HeLa cells (RCB0007) were obtained from Riken Cell Bank (Ibaraki, Japan) and cultured in the Eagle's MEM (Nissui, Tokyo, Japan) with 10% fetal bovine serum at 37°C in the presence of 5% CO_2. HeLa MT proteins were isolated from HeLa cells by the taxol-dependent method with some modifications (Hino *et al*., 2000). Tubulin was purified from whole pig cerebrum by the assembly-disassembly method followed by Whatman P-11 (Whatman, Clifton, NJ) column chromatography (Kotani *et al*., 1984). Sialidase (type X) from *Clostridium perfringens* and fetuin were purchased from Sigma Chemical (St. Louis, MO). RIBI Adjuvant System was from RIBI Immuno Chem Research (Hamilton, MO). PEG4000 was from Wako Pure Chemical (Osaka, Japan). Desialylation of fetuin was performed by treatment with 2M acetic acid at 80°C for 3 h.

Production and characterization of the monoclonal antibody SG3-1

Balb/c strain female mice were immunized with GM4 by intraperitoneal injection once a week. In the first immunization, RIBI Adjuvant System was injected with the immunogen as the adjuvant. After the forth immunization, the booster immunization was done three days before cell fusion. Spleen cells from the immunized mice were fused with mouse myeloma cells P3-X63-Ag8 using PEG 4000. Hybridomas were screened by the ability of their culture supernatants to bind to GM3, which shared in the epitope NeuAca2-3Gal with GM4, and one monoclonal antibody SG3-1 (IgM) was selected. Antigen binding ability of the SG3-1 was studied by ELISA using 96-well microtiter plate (Falcon No. 3912, Becton Dickinson, Bedford, MA, for glycolipid antigens: MS-8596F, Sumitomo Bakelite, Tokyo, Japan, for fetuin and asialo-fetuin) coated with various antigens as described (Kawamura *et al*., 1990).

SDS-PAGE and Immunoblotting

SDS-PAGE was carried out with 10% polyacrylamide slab gels as described previously (Murata-Hori *et al*., 1998). In some experiments, 8M urea-containing gel was used to separate α and β tubulin. For immunoblotting, samples were electrophoresed on SDS-PAGE and transferred to an Immobilon P-membrane (Millipore). The membranes were reacted with the SG3-1 and the blots were visualized with a Vectastain ABC kit (Vector Lab. Burlingame, CA) as described previously (Murata-Hori *et al*., 1998).

Sialidase treatment

Pig tubulin was separated on SDS-PAGE and transferred to an Immobilon P-membrane. The membranes were incubated at 37°C for 1 h in 66 mM sodium acetate buffer (pH 5.0), 1.3 mM $CaCl_2$ with or without sialidase from *Clostridium perfringens*. Then the membranes were subjected to immunoblotting with the SG3-1.

***N*-linked oligosaccharide analysis**

N-linked oligosaccharide analysis of pig tubulin was carried out according to Hotta *et al* (Hotta *et al*., 1985). The oligosaccharide fractions were collected by gel filtration on a Bio-Gel P-4 column (Bio-Rad Laboratories). Oligosaccharide fractions obtained were reductively aminated with 2-aminopyridine by the use of sodium cyanoborohydrate. The pyridylamino derivatives of oligosaccharides thus prepared were purified by gel filtration on a Sephadex G-15 column (Amersham Pharmacia). Then the PA-oligosaccharides were analyzed by HPLC on the HRC-ODS column (Shimadzu, Kyoto, Japan).

Immunofluorescence microscopy

HeLa cells grown on glass coverslips were fixed with methanol (–20°C) for 30 min. For some experiments, fixed cells were extracted with chloroform:methanol (1:1, by volume) to remove glycolipid antigens (Suzuki and Yamakawa, 1981). Cells were then washed twice in a phosphate buffer (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) followed by blocking with 3% BSA in PBS and incubated with hybridoma SG3-1 culture supernatant mixed with anti-α tubulin monoclonal antibody (clone DM1A, Seikagaku Kogyo, Tokyo, Japan). Cells were then washed three times in PBS and reacted with Alexa 568-conjugated goat anti-mouse IgM (Molecular Probes, Eugene, OR) mixed with the FITC-conjugated anti-mouse IgG FC (Cappel, Durham, NC). After further washing, the coverslips were mounted in Vectashield (Vector Lab.). Stained Cells were analyzed by using a LSM 410 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) with a 100× oil immersion objective lens.

RESULTS AND DISCUSSION

Characterization of the monoclonal antibody SG3-1

It has been reported that tubulin contains NeuAc and Gal as monosaccharides (Margolis *et al*., 1972). To analyze the glycosylation of tubulin, we used the monoclonal antibody SG3-1 that specifically recognizes the terminal NeuAcα2-3Gal structure. Firstly, the specificity of the monoclonal antibody SG3-1 was assessed by ELISA (Fig. 1A). The binding profile of the SG3-1 against glycolipids showed that the SG3-1 bound to NeuAcα2-3Galb1-1'Cer (GM4), NeuAcα2-3Galβ1-4Glcβ1-1'Cer (GM3), NeuAcα2-3Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-1'Cer (GM1b) and NeuAcα2-3Galβ1-4(Fucβ1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (sialyl Le^X) and did not bind to NeuAcα2-1'Cer (GM5), NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1'Cer (GD3), NeuAcα2-6Galβ1-4Glcβ1-1'Cer (2-6GM3), Galβ1-1'Cer (CMH) and Galβ1-4Glcβ1-1'Cer (CDH). Data for GM1b, sialyl Le^X, GD3 and CDH were not shown in Fig. 1A. All glycolipids recognized by the SG3-1 posses the NeuAcα2-3Gal structure at their non-reducing ends. In contrast, glycolipids that were negative for the SG3-1 do not posses the NeuAcα2-3Gal structure at their non-reducing ends. Interestingly, GD3, which posses the NeuAcα2-3Gal structure in the middle but not the end of saccharide sequence was negative for this antibody. Thus, we concluded that the SG3-1 recognizes the terminal NeuAcα2-3Gal structure of glycolipids. It should be noted that this antibody also recognizes the NeuAcα2-3Gal structure in glycoproteins. Fetuin, which is a glycoprotein and its carbohydrate structure has been well examined, possesses NeuAcα2-3Gal structure. The reactivity of the SG3-1 antibody to fetuin and its desialylated form, asialo-fetuin, was analyzed by ELISA (Fig. 1B). The antibody reacted with fetuin but not with asialo-fetuin. This result showed that the SG3-1 antibody reacts with not only glycolipid but also sialic acid in the glycoprotein.

Immunoblot analysis of tubulin molecules with the SG3-1

The reactivity of HeLa and pig tubulin with the SG3-1 was analyzed by immunoblot (Fig. 2). Pig tubulin (lanes 1 and 2) and HeLa MT proteins (lanes 3 and 4) were subjected to 8M urea-containing SDS-PAGE. Under this condition, β tubulin migrates faster than α tubulin. Then the blots were stained with Coomassie Brilliant Blue (CBB) (lanes 1 and 3) or probed with the SG3-1 (lanes 2 and 4). Both α and

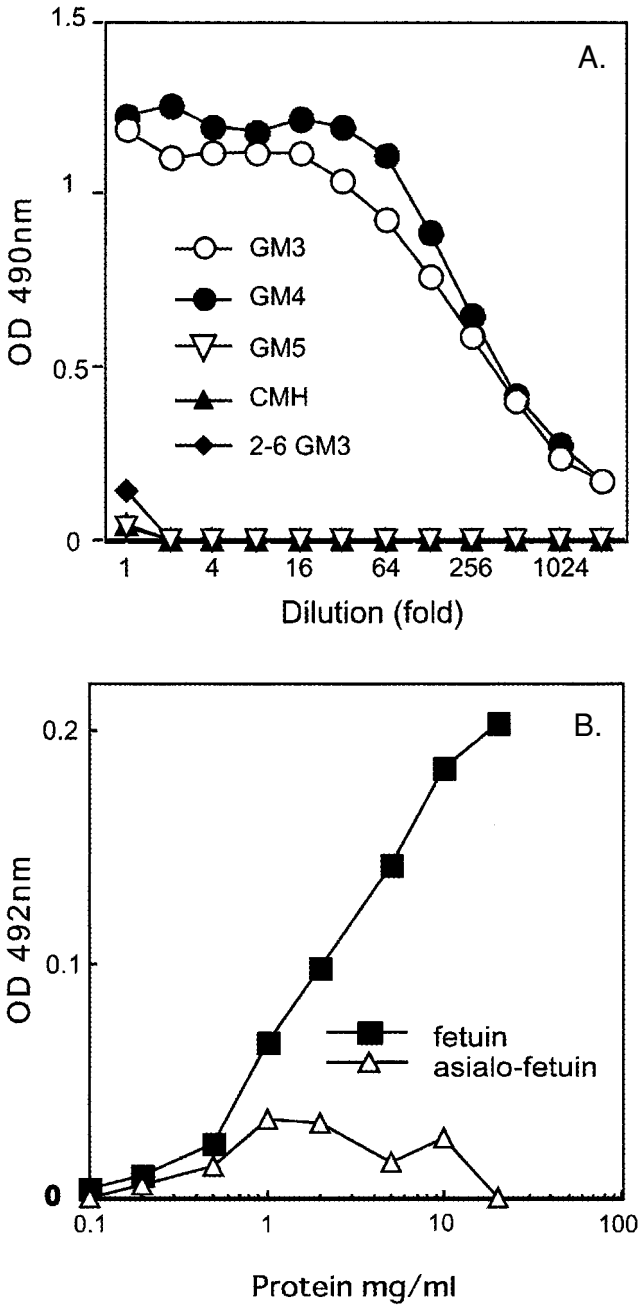

Fig. 1. Characterization of the monoclonal antibody SG3-1. A. ELISA of the SG3-1 antibody to glycolipids. See text for detail explanation for each glycolipid. Typical data from duplicate experiments are shown. B. ELISA of the SG3-1 to fetuin and asialo-fetuin. Typical data of two experiments.

β subunits of pig tubulin were recognized by the SG3-1 (lane 2). HeLa α and β tubulin were also immunoreactive to the SG3-1 (lane 4). The linkage between tubulin and the epitope was not sensitive to boiling in SDS-PAGE sample buffer or electrophoresis in 8M urea-containing SDS-PAGE. These results suggest that α and β tubulin are glycosylated with the glycan possessing the NeuAcα2-3Gal structure.

To confirm that the SG3-1 specifically recognizes sialic acid that glycosidically bound to tubulin molecules, pig tubulin was treated with sialidase which specifically hydrolyses terminal linked sialic acid from oligosaccharides (Fig. 3). To obtain tubulin enough for biochemical analysis, the pig tubulin but not the HeLa MTs was used in this experiment. Tubulin was subjected to SDS-PAGE, and transferred to PVDF membrane. On the PVDF membrane tubulin was treated with sialidase obtained from *Clostridium perfringens* (45mU/ml, lane 3; 450mU/ml, lane 4) and thus probed with the SG3-1 antibody. The immunostaining of tubulin with the SG3-1 was reduced at the treatment of 450mU/ml sialidase (lane 4). This result indicates that the SG3-1 recognizes glycosidically bound sialic acid of tubulin molecules. The effective concentration of sialidase (450 mU/ml) was 100 times higher than that was suggested by the manufacturer (Sigma). In the work by Margolis *et al.* (1972), tubulin was also treated with 100 times excess of sialidase from *Vibrio cholerae*. These results might suggest that high concentration of sialidase is required to release sialic acid from tubulin molecules.

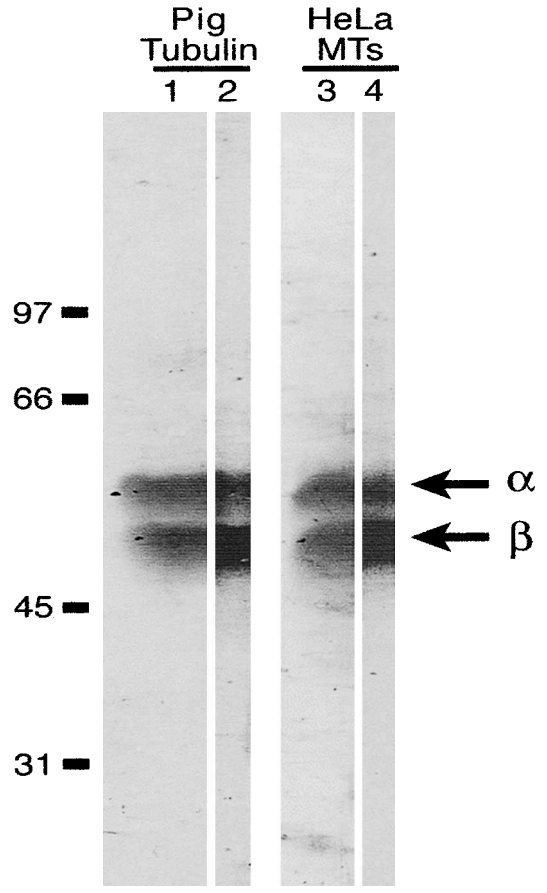

Fig. 2. Immunoblot analysis of pig tubulin and HeLa MT proteins. Pig tubulin (lanes 1 and 2) and HeLa MT proteins (lanes 3 and 4) were subjected to SDS-PAGE, transferred to PVDF membrane, and stained with Coomassie brilliant blue (CBB) (lanes 1 and 3) or probed with the SG3-1 antibody (lanes 2 and 4). Arrows indicate α and β tubulin. The positions of molecular mass markers are indicated with molecular mass in kilodaltons. Typical data of three experiments.

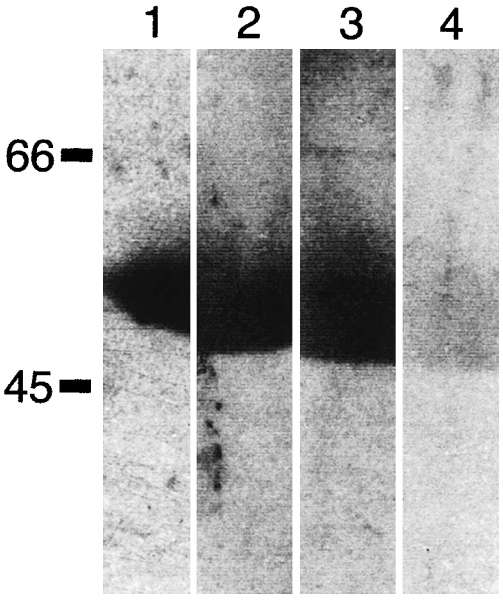

Fig. 3. Sialidase treatment of pig tubulin. Pig tubulin was subjected to SDS-PAGE, transferred to PVDF membrane, and treated with sialidase from *Clostridium perfringens* at 0 mU/ml (lane 2), 45 mU/ml (lane 3) and 450 mU/ml (lane 4) as described in the Materials and Methods. Then the blots were stained with CBB (lane 1) or proved with the SG3-1 antibody (lanes 2-4). Typical data of three experiments.

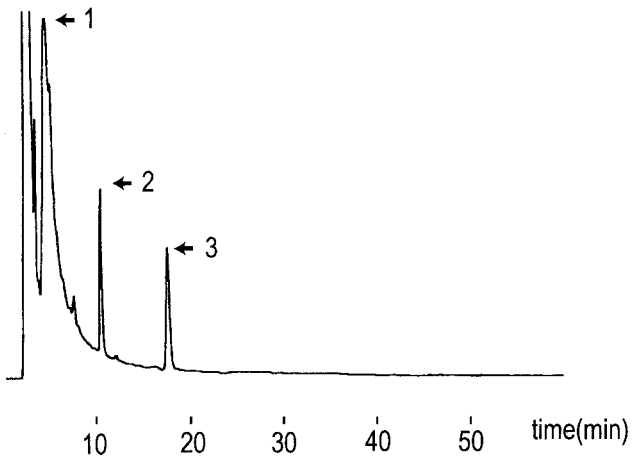

Fig. 4. Profiles of brain tubulin *N*-linked oligosaccharides. Three major *N*-linked oligosaccharides are numbered as 1, 2 and 3.

N-linked oligosaccharide analysis of tubulin molecules

N-linked oligosaccharides of tubulin were analyzed by HPLC. *N*-linked oligosaccharides were released from pig tubulin and analyzed by HPLC as described in the Materials and Methods. The HPLC profile showed three major *N*-linked oligosaccharides of pig tubulin, which were numbered as 1, 2 and 3 (Fig. 4). Although the features of these oligosaccharides were not determined, this result showed a possible existence of *N*-linked oligosaccharides in pig tubulin. Since α and β tubulin contain one and three potential *N*-glycosylation sites, respectively, tubulin may be glycosylated at these sites if the glycosylation is carried out by known glycosylation enzymes. From this point of view, we need to investigate the *N*-glycosylated site(s) of α and β tubulin.

Colocalization of sialic acid with MTs

Finally, the localization of the NeuAcα2-3Gal structure in HeLa cell was analyzed by immunofluorescence microscopy (Fig. 5). Confocal images showed that HeLa cells were positively stained with the SG3-1, showing filamentous structure originating near the nucleus and extending out towards the periphery of the cell (panel A). These filamentous patterns are similar to those of MTs or intermediate filaments (IFs). It has been reported that some glycolipids localize with MTs (Sakakibara *et al.*, 1981a, b; Nagai and Sakakibara, 1982) and IFs (Gillard *et al.*, 1992; Kotani *et al.*, 1994) in the non-extractable conditions such as formaldehyde fixation. In the extractable conditions like methanol fixation, the colocalization of these glycolipids with cytoskeletons disappears. Since the cells were fixed in methanol in our experiments, glycolipid antigens that non-covalently bind

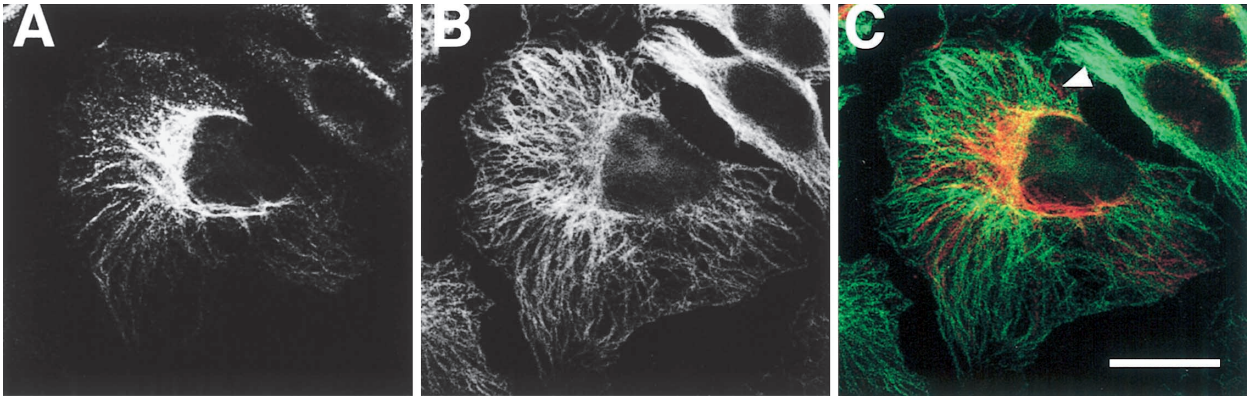

Fig. 5. Confocal immunofluorescence microscopy of HeLa cells. Cells were fixed with methanol and double-stained with SG3-1 (panel A) and anti-α tubulin antibody (panel B). Merged images are also shown in panel C. An arrowhead in panel C indicates the filamentous structure which is recognized by the SG3-1 (red) but not with anti-α tubulin antibody (green). Scale bar indicates 10 μm. Typical data of three experiments.

to proteins might have been extracted. The filamentous patters (panel A) were observed after methanol fixation, and even after lipid extraction with the mixture of chloroform and methanol (data not shown), suggesting that the antigen recognized by the SG3-1 is probably the glycoprotein rather than the glycolipid. To identify these filamentous structures as MTs, HeLa cells were double-stained with the SG3-1 (panel A) and monoclonal anti-α tubulin antibody (panel B). The filamentous staining with the SG3-1 was overlapped with MTs obviously in the vicinity of the nucleus and to lesser extent with peripheral MTs (panel C). Interestingly, a few SG3-1-labeld filamentous structures were not stained with anti-a tubulin antibody (arrowhead in panel C). We need further investigations to clarify whether the filamentous structures are identical with other cytoskeletal structures such as IFs and actin filaments.

Conclusion and perspective

The present *in vitro* and *in vivo* data indicate a possible glycosylation of the α and β tubulin by sialyloligosaccharides. The α and β tubulin plays critical roles in a large number of cellular activities (Hyams and Lloyd, 1993). Glycosylation may be involved in the mechanism that regulates the functions of the α and β tubulin in cooperation with other post-translational modifications.

Sialylation is catalyzed by sialyltransferases, a family of more than 15 enzymes which transfer the sialic acid from CMP-sialic acid to the non-reducing terminal positions of oligosaccharide in glycoproteins and glycolipids (Tsuji, 1996). Since all sialyltransferases function in the lumen of the Golgi apparatus, α and β tubulin are thought to be sialylated in the Golgi apparatus. Interestingly, α and β tubulin were detected in the lumen of rough microsome obtained from pig brain (Gilbert and Strocchi, 1986), suggesting that some portions of the α and β tubulin are translated in endoplasmic reticulum (ER). Recent works have also shown that there is a system, which retrogradely transports proteins from ER to cytosol (Parodi, 2000; Spiro, 2000; Lehrman, 2001). If we take all results of the present study and the previous works into consideration, we would suggest a model that parts of the α and β tubulin molecules are translated and glycosylated in the ER-Golgi region and then retrogradely transported to the cytosol.

The number of reports showing the existence of the glycoproteins (Hart *et al*., 1989; Schafer and Sorrell, 1993; Wang *et al*., 1996; Goletz *et al*., 1997; Sassi *et al*., 2001) and glycolipids (Sakakibara *et al*., 1981a, b; Nagai and Sakakibara, 1982; Symington *et al*., 1987; Gillard *et al*., 1992; Kotani *et al*., 1994) in cytosol is increasing. The presence of galectins (Cooper and Barondes, 1999; Hughes, 1999) and glycosidases such as endo-glucosaminidase (Pierce *et al*., 1979), peptide *N*-glycanase (Suzuki *et al*., 1994), chitobiase (Cacan *et al*., 1996; Kato *et al*., 1997), mannosidase (Grard *et al*., 1994) and sialidase (Miyagi *et al*., 1993) in cytosol consistently supports the existence of the cytosolic glycoconjugates. However, little is known about the role(s) of the cytosolic glycoconjugates. It is interesting to note that some of them are associated with cytoskeletons. Numbers of cytoskeletal proteins including MT associated proteins, cytokeratins and actin binding proteins are *O*-GlcNAcylated (Arnold and Hart, 1999). Tau, one of the microtubule associated proteins, is glycosylated in Alzheimer's disease brain (Wang *et al*., 1996; Takahashi *et al*., 1999). Cytokeratins are keratan-sulfated (Schafer and Sorrell, 1993) and glycosylated with saccharides containing terminal α1-3GalNAc (Goletz *et al*., 1997). Glycolipids such as globoside and gangliosides are colocalized with the MTs (Sakakibara *et al*., 1981a, b) and IFs (Gillard *et al*., 1992; Kotani *et al*., 1994). Galectins are associated with actin (Chiu and O'Keefe, 1992; Joubert *et al*., 1992). Our present data showed that α and β tubulin are glycosylated. It has been observed that cytoskeletons such as MTs (Varki *et al*., 1999) and IFs (Gillard *et al*., 1996) are involved in the metabolism of glycoconjugates. These observations illustrate the importance of the cytoskeletons in elucidating the function of glycoconjugates in cytosol. Further studies on the function of glycosylation of α and β tubulin would provide basic information to understand the biological significance of cytosolic glycoconjugates.

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