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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 (Pature-Lafanechere et al., 1991), polyglycylation (Redeker et al., 1994), palmitoylation (Caron, 1997), and glycossylation (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 [14C]-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 NeuAcα2-3Galβ1-3GlcNAcβ1-4Glcβ1-4Glcβ1-1′Cer (GM1b), NeuAcα2-3Galβ1-4Glcβ1-1′Cer (GM3), NeuAcα2-3Galβ1-1′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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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 NeuAca2-3Gal 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 NeuAca2-3Galb1-1'Cer (GM4), NeuAca2-3Galβ1-4Glcβ1-1'Cer (GM3), NeuAca2-3Galβ1-4Glcβ1-3Galβ1-4Glcβ1-1'Cer (GD3) and NeuAca2-3Galβ1-4Glcβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (CDH) and did not bind to NeuAca2-1'Cer (GM5), NeuAca2-3Galβ1-4Glcβ1-1'Cer (GD3), NeuAca2-3Galβ1-4Glcβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (GD3), NeuAca2-3Galβ1-4Glcβ1-1'Cer (CMH) and Galβ1-4Glcβ1-1'Cer (CDH). Data for GM1b, sialyl Le\(^\text{X}\) and GD3 and CDH were not shown in Fig. 1A. All glycolipids recognized by the SG3-1 possess the NeuAca2-3Gal structure at their non-reducing ends. In contrast, glycolipids that were negative for the SG3-1 do not possess the NeuAca2-3Gal structure at their non-reducing ends. Interestingly, GD3, which possesses the NeuAca2-3Gal 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 NeuAca2-3Gal structure of glycolipids. It should be noted that this antibody also recognizes the NeuAca2-3Gal structure in glycoproteins. Fetuin, which is a glycoprotein and its carbohydrate structure has been well examined, possess NeuAca2-3Gal 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
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 antibody. 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.
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. 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. 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.](https://bioone.org/journals/Zoological-Science)

![Fig. 4. Profiles of brain tubulin N-linked oligosaccharides. Three major N-linked oligosaccharides are numbered as 1, 2 and 3.](https://bioone.org/journals/Zoological-Science)

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 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.

![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.](https://bioone.org/journals/Zoological-Science)
to proteins might have been extracted. The filamentous patterns (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-labeled filamentous structures were not stained with anti-α 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 (Tsui, 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; Goetz 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, cytokehcinases 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 (Goetz 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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