

High Molecular Weight Microtubule-associated Proteins Contain *O*-Linked *N*-Acetylglucosamine*

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We have examined the post-translational modification of high molecular weight microtubule-associated proteins (MAPs) and have shown that MAP1, MAP2, and MAP4 are glycosylated. The presence of carbohydrate residues on these proteins was indicated by labeling with biotin hydrazide following periodate oxidation, a specific and well established method for detecting saccharide moieties on proteins. Both MAP2 and MAP4 were also labeled *in vitro* by UDP-³H]galactose in the presence of galactosyltransferase. Labeling by galactosyltransferase indicated that MAP2 and MAP4 contained terminal nonreducing GlcNAc residues, and they appeared to be *O*-linked to the proteins as shown by their sensitivity to β -elimination. Chromatographic analysis showed that the GlcNAc residues were directly linked to the proteins as monosaccharides. Thus, we have added MAP2 and MAP4 to the list of intracellular *O*-GlcNAc-modified proteins, which includes other cytoskeletal proteins such as cytokeratins 8, 13, and 18 and neurofilament proteins NF-L and NF-M. We further characterized the *O*-GlcNAc modification of MAP2, and stoichiometric analysis indicated that nearly 10% of the MAP2 isolated from rat brain is modified by *O*-GlcNAc. However, this estimate is thought to reflect the minimal level of *O*-GlcNAc modification present on MAP2. We have also shown that both the *O*-GlcNAc and biotin hydrazide-reactive carbohydrate moieties are located on the projection domain of MAP2. Three *O*-GlcNAc-containing peaks were observed following fast protein liquid chromatography of a tryptic digest of MAP2, suggesting that multiple modification sites exist. The specific modification sites and functional significance of the *O*-GlcNAc glycosylation on the high *M_r* MAPs remain to be determined.

Microtubules (MTs)¹ are one of the major components of the cytoskeleton and play an important role in the organization of the cytoplasm. As an example, disruption of MTs by various MT-stabilizing agents results in major changes in cytoplasmic organization, including collapse of intermediate filaments and redistribution of the Golgi apparatus and the endoplasmic

reticulum (1). MTs are also involved in such diverse cellular functions as maintenance of cell shape, movement of eukaryotic cilia and flagella, formation of the mitotic spindle, and regulation of organelle distribution and vesicle movements (2). Regulation of the dynamic properties of MTs is thought to play a role in many of these processes (3).

A group of proteins that bind to MTs *in vivo* and copurify with MTs, collectively defined as microtubule-associated proteins (MAPs), modulate MT dynamics and function. MAPs can be categorized into two major classes according to their primary function, *i.e.* motor proteins, which include the kinesin superfamily and the dynein family, and non-motor proteins, which are traditionally further divided into the high molecular weight MAPs (including MAP1A, MAP1B, MAP2, and MAP4) and the lower molecular weight tau proteins. Among the identified *in vivo* functions of MAPs in neuronal cells, kinesin and cytoplasmic dynein are responsible for anterograde and retrograde axonal transport, respectively, while tau protein and MAP2 appear to be required for initial neurite growth as shown using antisense mRNA (4, 5). It is well known that the functions of MAPs can be regulated through phosphorylation. For example, phosphorylation of MAPs can alter their affinity for MTs *in vitro* and affect their ability to stabilize MTs (6). Furthermore, the phosphorylation of MAP4 has been coupled to the onset of mitosis *in vivo*, and this phosphorylation may play a functional role in regulating MT dynamics during the interphase to mitosis transition (7). Post-translational modification of MAPs other than phosphorylation may also play a role in regulating MAP function; however, little is known about other potential forms of MAP modification.

During the past decade, a unique glycosylation of cytoplasmic and nuclear proteins has been characterized and identified as an *O*-GlcNAc modification. This modification is composed of a single *N*-acetylglucosamine *O*-linked to either a serine or threonine residue (8, 9). Virtually all of the *O*-GlcNAc-modified proteins identified to date are also phosphorylated, and they form reversible multimeric complexes in a regulated manner. Analogous to reversible phosphorylation reactions catalyzed by specific kinases and phosphatases, the corresponding *O*-GlcNAc-transferases and glycosidases have also been identified (10, 11). It has been proposed that *O*-GlcNAc modifications may play a regulatory role similar to that of phosphorylation. A recent report has shown that *O*-GlcNAc residues on the associated 67-kDa protein (p67) of eukaryotic initiation factor 2 (eIF-2) are indispensable for its ability to protect eIF-2 from inactivation following phosphorylation by eIF-2 kinase (12). Interestingly, this rapidly growing list of *O*-GlcNAc-modified proteins includes several cytoskeletal components, namely, cytochrome 13 (13), cytokeratins 8 and 18 (14), and neurofilaments (15). This prompted us to ask if glycosylation, *O*-GlcNAc modification in particular, could be another general means of post-translational modification of MAPs.

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¹ The abbreviations used are: MTs, microtubules; MAP, microtubule-associated protein; eIF-2, eukaryotic initiation factor 2; FPLC, fast protein liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

We report here that the high M_r MAPs are glycosylated as indicated by their labeling with biotin hydrazide following periodate oxidation. The biotin hydrazide labeling method is a common technique used to detect saccharide moieties on proteins (16). More important, both MAP2 and MAP4 contain *O*-linked *N*-acetylglucosamine residues in the monosaccharide form, adding these MAPs to the growing list of *O*-GlcNAc-modified proteins. We have further shown that the biotin hydrazide-labeled carbohydrate and the *O*-GlcNAc modification are both localized to the MAP2 projection domain. Analysis of MAP2 tryptic digests indicates that there may be several *O*-GlcNAc modification sites on MAP2. Our findings have revealed a previously unknown form of post-translational modification on high M_r MAPs, which may prove to be critical in understanding their intracellular functions, especially their interactions with MTs and other cellular components.

EXPERIMENTAL PROCEDURES

Materials—[6-³H]Glucosamine (27 Ci/mmol) was obtained from ICN Biomedicals, Inc. The ECL glycoprotein detection system was obtained from Amersham Corp., and the *O*-GlcNAc detection kit was purchased from Oxford Glycosystems. Bovine milk galactosyltransferase, UDP-galactose, and UDP-[6-³H]galactose (15.3 Ci/mmol) were obtained from Sigma. ENTENSIFY™ universal autoradiography enhancer used in fluorography was from DuPont NEN. The GlcNAc β 1-4GlcNAc standard was obtained from Seikagaku America, Inc. BCA protein assay reagents were purchased from Pierce. Human plasma thrombin was obtained from Calbiochem. The PeRPC HR 5/5 column and FPLC system were obtained from Pharmacia Biotech Inc.

Preparation of Microtubule-associated Proteins—Fresh rat brains were homogenized in 1.5 volumes of cold PEM buffer (0.1 M PIPES/NaOH, 1 mM EGTA, 1 mM MgSO₄, pH 6.6), followed by centrifugation at 30,000 $\times g$ in a JA-20 rotor (Beckman J2-21 centrifuge) for 15 min at 4 °C. The supernatant was centrifuged again at 180,000 $\times g$ in a Ti-60 rotor (Beckman L8-M ultracentrifuge) for 90 min at 4 °C. The supernatant from the second centrifugation was recovered, and Taxol and GTP were added to final concentrations of 20 μ M and 1 mM, respectively. The sample was warmed to 37 °C for 15 min, followed by centrifugation at 37 °C at 30,000 $\times g$ in a JA-20 rotor for 30 min. The rat MT pellet was washed in warm PEM buffer containing 20 μ M Taxol and 1 mM GTP and resuspended in PEM buffer.

Rat MAP2 and tubulin were purified from the rat MT pellet by adding NaCl to a final concentration of 0.35 M. After incubation in a boiling water bath for 8 min, the sample was centrifuged at 15,800 $\times g$ (Brinkmann Instruments Model 5402 microcentrifuge) at 4 °C for 15 min. Heat-stable MAP2 was recovered in the supernatant. Alternatively, after addition of NaCl to 0.35 M, the Taxol-stabilized rat MTs were incubated at 37 °C for 15 min, followed by centrifugation at 15,800 $\times g$ at 37 °C for 15 min. The salt-extracted pellet was resuspended in PEM buffer and used as Taxol-stabilized tubulin. HeLa MAP4 was kindly provided by Yunhi Choi of this laboratory and was purified essentially as described by Vallee and Collins (17).

Glycoprotein Detection—Carbohydrates on MAPs were labeled using the ECL glycoprotein detection system following the manufacturer's protocol with some modifications. Briefly, samples were separated by SDS-PAGE and transferred to nitrocellulose membranes, followed by reduction with 1 mg/ml sodium borohydride in TBS buffer (10 mM Tris base, pH 7.4, 154 mM NaCl) to remove endogenous reacting groups (six times, 15 min each time). Sodium metaperiodate was used to oxidize the vicinal hydroxyls of carbohydrate moieties, resulting in aldehyde generation. The samples were subsequently treated with biotin hydrazide to incorporate biotin into the oxidized carbohydrate. Finally, the biotin was detected by horseradish peroxidase-conjugated streptavidin using enhanced chemiluminescence (ECL).

Galactosyltransferase Labeling—Bovine milk galactosyltransferase was autogalactosylated prior to use (18). MAP samples were usually incubated at 0 °C for 3 h in labeling buffer (10 mM HEPES/NaOH, 10 mM galactose, 5 mM MnCl₂, pH 7.3) containing 1 μ Ci of UDP-[³H]galactose and 30 million units of β -galactosyltransferase in a final volume of 45 μ l. After the reactions were stopped by addition of EDTA to 10 mM, a portion of the samples were separated by SDS-PAGE and visualized by fluorography. Alternatively, the remaining samples were incubated at 37 °C overnight with β -elimination buffer (1 M NaBH₄, 0.1 M NaOH), β -galactosidase, or distilled water and then analyzed by adsorption to polyvinylidene difluoride membrane and assayed using the *O*-GlcNAc

detection kit according to the manufacturer's instructions.

Carbohydrate Composition Analysis—Rat MAP2 and ovalbumin were labeled with galactosyltransferase as described above. Samples were then dialyzed against 0.2 M NaCl to remove free UDP-[³H]galactose and lyophilized. Samples were incubated either with 300 μ l of β -elimination buffer at 37 °C for 18 h followed by neutralization with ice-cold 4 M acetic acid or with distilled water as a control. The reaction mixtures were lyophilized and washed twice with 1 M acetic acid/methanol in the presence of excess galactose to remove residual borate. Finally, samples were applied to a Bio-Gel P-4 column (-250 mesh, 0.9 \times 50 cm) at 16 ml/h in 0.2 M ammonium acetate. The column V_0 and V_f volumes were established by using cytochrome *c* and galactose, respectively. [³H]Glucosamine and GlcNAc β 1-4GlcNAc were also used to calibrate the column. Galactose was detected by phenol/sulfuric acid assay (19). After separation on the P-4 column, an equal volume was removed from each eluted fraction, and ³H was measured in a scintillation counter. The mole concentration of rat MAP2 was calculated from its absorbance at 562 nm using the BCA protein assay. After the galactosyltransferase labeling reaction, the incorporated radioactivity was detected by polyvinylidene difluoride membrane adsorption assay, and the total moles of conjugated [³H]galactose was deduced using the specific activity of UDP-[³H]galactose.

Thrombin Digestion and Microtubule Binding Assay—Rat MAP2 was digested by thrombin according to Joly *et al.* (20) at a final concentration of 32 units/ml, and reactions were stopped by addition of phenylmethylsulfonyl fluoride to 1 mM. Part of the reaction mixture was separated by SDS-PAGE and analyzed by the glycoprotein detection system; the remaining part was tested for microtubule binding ability as described below. Thrombin-digested MAP2 was mixed with Taxol-stabilized rat tubulin. After incubation at 37 °C for 15 min in the presence of 1 mM GTP and 20 μ M Taxol, the samples were centrifuged at 10,000 $\times g$ for 10 min. The pellets were resuspended in SDS sample buffer after the supernatant had been collected. The whole mixture, supernatant, and pellet were then analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Some rat MAP2 was labeled by [³H]galactose, as described earlier, prior to thrombin digestion under the same conditions. These samples were then separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and analyzed by fluorography.

Tryptic Mapping of MAP2—Rat brain MAP2 was labeled with [³H]galactose as described above and dialyzed against 0.2 M NaCl followed by distilled water. The sample was then lyophilized and processed for trypsin digestion at an enzyme/substrate ratio of 1:40 (w/w) at 37 °C for 24 h. The peptides were then separated on a PeRPC HR 5/5 reverse-phase column using the Pharmacia FPLC system. The peptides were bound to the column in 0.1% trifluoroacetic acid in FPLC-grade water and eluted with an increasing gradient of acetonitrile. A constant level of 0.1% trifluoroacetic acid was present in all solvent mixtures. Elution of peptides was monitored by A_{215} , and the radioactivity in each fraction was then analyzed by liquid scintillation counting.

SDS-PAGE and Immunoblot Analysis—Samples were analyzed by SDS-PAGE and immunoblotting as described earlier (21) with the following modifications. Gradient gels (4–12 or 7–17% acrylamide) were used for gel electrophoresis, and proteins were transferred to nitrocellulose paper with a 0.2- μ m pore size at 250 mA for 1 h in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, 0.05% SDS); a second transfer was at 150 mA for 15 min using transfer buffer without SDS. Nitrocellulose paper was incubated in 10% (v/v) heat-inactivated horse serum in TBS buffer for 1 h to block nonspecific binding sites, rinsed with TBS buffer, and incubated with various primary antibodies for 1 h. After washing in TBS buffer, the transfer was incubated with peroxidase-conjugated goat anti-mouse antibody, washed, and visualized by ECL.

RESULTS

High M_r MAPs Are Glycosylated—In a recent report, tau protein, a low molecular weight MAP, was shown to be nonenzymatically glycosylated in the brain tissue of Alzheimer's disease patients (22, 23). In addition, it has also been reported that claustrin, a chicken brain homologue of MAP1B, is a keratan sulfate-containing protein (24). Furthermore, it was shown that rat brain MAP1B is also sensitive to keratanase digestion, which hydrolyzes the β -galactosidic linkages present in keratan sulfate. These reports suggested that post-translational modifications other than phosphorylation might also occur on MAPs.

To determine whether glycosylation might be another com-

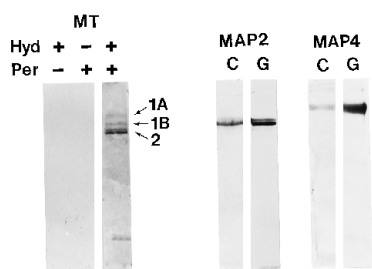


FIG. 1. Glycoprotein detection using biotin hydrazide/streptavidin following metaperiodate oxidation. Rat brain MTs (*MT*) were separated on a 4% urea-SDS gel and transferred to nitrocellulose. Nitrocellulose transfers were then analyzed using the glycoprotein detection kit as described under "Experimental Procedures." Control experiments were done by omitting either the sodium metaperiodate oxidation step (*-Per*) or the biotin hydrazide conjugation step (*-Hyd*). The positions of MAP1A, MAP1B, and MAP2 as determined by Coomassie staining and immunoblot analysis (data not shown) are indicated as *1A*, *1B*, and *2*, respectively. Boiled MAP2 and boiled MAP4 were separated by 4% urea-SDS-PAGE and 7.5% SDS-PAGE, respectively. They were either stained with Coomassie Brilliant Blue (*C*) or analyzed by the glycoprotein detection kit (*G*). This highly specific reaction indicated that MAP1, MAP2, and MAP4 contained carbohydrate moieties.

mon post-translational modification of high M_r MAPs, we employed a well established glycoprotein detection method (16). In essence, this approach is based upon the oxidation of the vicinal hydroxyls on carbohydrate moieties by periodate and the subsequent formation of aldehyde groups. The newly generated aldehyde groups are available to react with various hydrazide probes, the binding of which can be detected following completion of the reaction. Using a biotin hydrazide/streptavidin detection system, we observed that MAP1A, MAP1B, and MAP2 present in rat brain MT samples were all labeled following periodate oxidation (Fig. 1, *MT*). The specificity of this reaction was confirmed by the negative results obtained in the experiments omitting either periodate oxidation (Fig. 1, *MT*, *-Per*) or biotin hydrazide conjugation (Fig. 1, *MT*, *-Hyd*). Isolation of heat-stable MAP2 following boiling of the rat brain MT preparation confirmed the reaction of this MAP with the biotin hydrazide-labeled carbohydrate detection system (Fig. 1, *MAP2*). These results indicate that brain high M_r MAPs contain some form of carbohydrate modification. Furthermore, MAP4 isolated from HeLa MT preparations was also strongly labeled by biotin hydrazide (Fig. 1, *MAP4*). Thus, the presence of carbohydrate residues on high M_r MAPs was not restricted to brain samples, but also included the major MAP found in non-neuronal cells.

MAP2 and MAP4 Contain O-Linked Terminal Nonreducing N-Acetylglucosamine—Although cytoplasmic proteins rarely contain complex carbohydrate modifications, recent evidence shows that there is a unique *O*-GlcNAc modification found almost exclusively on cytoplasmic and nuclear proteins, including some cytoskeletal components (9). In an effort to determine which carbohydrate components exist on MAPs, we examined high M_r MAPs following a standard approach used for the detection of *O*-GlcNAc.

The isolated MAPs were labeled using a UDP- ^3H galactose/galactosyltransferase system that specifically transfers ^3H galactose to a terminal nonreducing *N*-acetylglucosamine. Labeled samples were then separated by SDS-PAGE and stained with Coomassie Brilliant Blue, and the stained samples were subjected to fluorography. Ovalbumin containing *N*-linked terminal nonreducing *N*-acetylglucosamine was used as a control (Fig. 2A, lanes 1 and 4). Our results showed that both mitotic MAP4 and interphase MAP4 isolated from HeLa cells were labeled (Fig. 2A, lanes 2 and 5 and lanes 3 and 6,

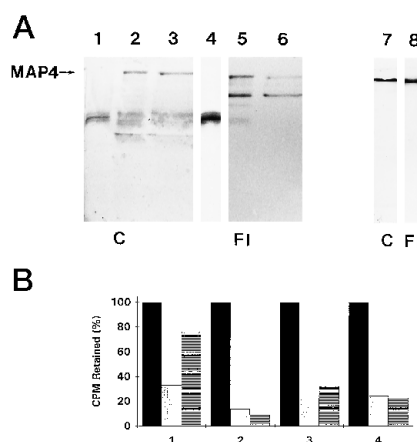


FIG. 2. Galactosyltransferase labeling and glycosidic linkage analysis of MAP2 and MAP4. Samples were galactosyltransferase-labeled as described under "Experimental Procedures." A, labeled samples were separated by either 7–17% (lanes 1–6) or 7% (lanes 7 and 8) SDS-PAGE and stained by Coomassie Brilliant Blue (*C*), followed by fluorography analysis (*F*) of the same gels. Lanes 1 and 4, ovalbumin; lanes 2 and 5, mitotic MAP4; lanes 3 and 6, interphase MAP4; lanes 7 and 8, rat brain MAP2. B, labeled ovalbumin (1), MAP2 (2), mitotic MAP4 (3), and interphase MAP4 (4) were incubated at 37 °C for 18 h with β -galactosidase (□), with β -elimination buffer (▨), or with distilled water (■) as control. Radioactivity retained after various treatments was plotted as a percentage of the total radioactivity in control samples. Note the high sensitivity of MAP2 and MAP4 to β -elimination buffer as compared with ovalbumin.

respectively) and that rat brain MAP2 was also heavily labeled (lanes 7 and 8) by ^3H galactose. Rat brain MAP1 was only weakly labeled by ^3H galactose (data not shown). In addition to the MAP4 band, another heavily labeled band was present in both of the MAP4 samples. These additional labeled bands were minor Coomassie staining bands, suggesting that this band could be a breakdown product of MAP4, or alternatively, it could be another heat-stable HeLa MAP that contains a high amount of *N*-acetylglucosamine. Interestingly, both MAP4 and the lower molecular weight protein showed a higher degree of labeling in the mitotic sample, suggesting a potential cell cycle-dependent regulation of this glycosylation.

To prove that the label was covalently linked to the protein via *O*-glycosidic bonds, equal amounts of the labeled samples were incubated with either β -galactosidase or β -elimination buffer overnight. The radioactivity retained by the samples following this treatment was analyzed after binding of the protein to polyvinylidene difluoride and was compared with that of control samples that were incubated with distilled water only (Fig. 2B). β -Galactosidase, an enzyme that specifically cleaves terminal nonreducing galactose from carbohydrate, removed at least 70% of the incorporated radioactivity from MAP2, mitotic MAP4, and interphase MAP4. Furthermore, β -elimination buffer, which cleaves *O*-linked carbohydrate from protein but leaves *N*-linked carbohydrate intact, removed 90% of the radioactivity from MAP2 and 70% from mitotic MAP4 and interphase MAP4. These data indicate that MAP2, mitotic MAP4, and interphase MAP4 all contain *O*-linked terminal nonreducing *N*-acetylglucosamines. On the other hand, ovalbumin showed high sensitivity to β -galactosidase, but resistance to β -elimination buffer, as expected for a protein containing *N*-linked terminal *N*-acetylglucosamine (Fig. 2B).

MAP2 Contains GlcNAc Directly O-Linked to the Protein—Since the rat brain MAP2 sample was relatively pure and the labeling was highly specific (Fig. 2A, lanes 7 and 8), further characterization of the carbohydrate associated with MAPs was focused on MAP2. To ascertain that MAP2 contained *O*-GlcNAc directly linked to the protein, we needed to provide

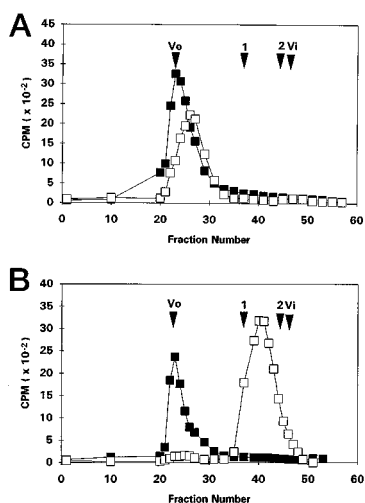


FIG. 3. Chromatographic analysis of β -elimination products from galactosyltransferase-labeled MAP2 and ovalbumin. Galactosyltransferase-labeled ovalbumin (*A*) and MAP2 (*B*) were analyzed by Bio-Gel P-4 chromatography following incubation with (□) or without (■) β -elimination buffer. The column V_0 and V_i volumes were established by using cytochrome *c* and galactose, respectively. Glucosamine (*2*) and GlcNAc β 1-4GlcNAc (*1*) were also used to calibrate the column. The volume of each fraction is 0.63 ml. The β -elimination product of galactosyltransferase-labeled MAP2 migrated as a single peak between glucosamine and GlcNAc β 1-4GlcNAc (*B*), as expected for an *O*-GlcNAc-modified protein.

evidence that the labeled carbohydrate was a monosaccharide instead of an *O*-linked polysaccharide containing terminal GlcNAc residues. Briefly, MAP2 was labeled with galactosyltransferase and [3 H]galactose, dialyzed, and incubated either with water as control or with β -elimination buffer to remove labeled carbohydrates. The samples were then analyzed by high resolution Bio-Gel P-4 chromatography. If the original carbohydrate residue was a monosaccharide, the expected β -elimination product would be [3 H]Gal β 1-4-*N*-acetylglucosaminitol. This product should migrate slower on the Bio-Gel P-4 column than the disaccharide control GlcNAc β 1-4GlcNAc since two simple monosaccharides behave as one GlcNAc on the Bio-Gel P-4 column (25). On the other hand, if the original carbohydrate contains a terminal GlcNAc in addition to one or more monosaccharide residues, the [3 H]galactose-containing β -elimination product would migrate at least at the same position as GlcNAc β 1-4GlcNAc, if not faster. A labeled complex carbohydrate containing a terminal GlcNAc residue would be excluded from the column resin and migrate with the void volume.

Ovalbumin was used as a control, and as expected, β -elimination did not significantly change the elution position of the [3 H]galactose-containing peak (Fig. 3*A*). As shown in Fig. 3*B*, the radioactivity associated with the MAP2 control sample also eluted at the V_0 position. However, the [3 H]galactose-labeled fraction obtained after β -elimination of the MAP2 sample migrated as a single peak between GlcNAc β 1-4GlcNAc and glucosamine. These results exclude the possibility that the labeled terminal GlcNAc on MAP2 was associated with a complex polysaccharide moiety. We thus conclude that the original carbohydrate moiety on MAP2 is an *O*-linked *N*-acetylglucosamine monosaccharide. Similar results were obtained with [3 H]galactose-labeled MAP4 (data not shown).

To determine the extent of *O*-GlcNAc modification, identical amounts of MAP2 were galactosyltransferase-labeled for different time periods at 0 °C, and the total incorporated radioactivity was measured (Fig. 4*A*). The reaction proceeded rapidly, and by 180 min, all of the available GlcNAc residues were

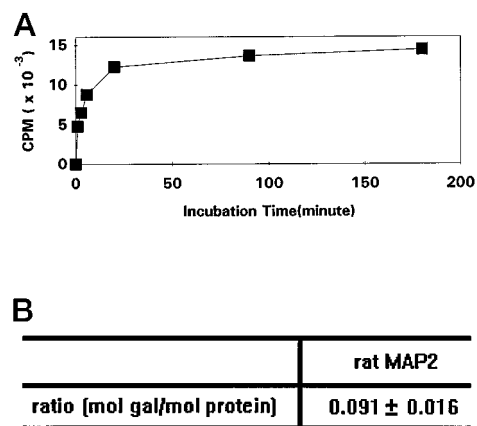


FIG. 4. Stoichiometry of *O*-GlcNAc-modified MAP2. An equal amount of rat brain MAP2 was galactosyltransferase-labeled for 1, 3, 6, 15, 90, or 180 min, and the total incorporated radioactivity was detected by polyvinylidene difluoride adsorption assay. The 180-min samples were used to deduce the mole ratio of *O*-GlcNAc to protein. *A*, a representative graph of galactosyltransferase labeling reactions on MAP2; *B*, the average mole ratio of incorporated [3 H]galactose to MAP2, calculated from eight experiments.

labeled. Higher temperature (37 °C) or longer incubation time (up to 5 h) did not generate a significant difference in the total incorporated radioactivity (data not shown). The 180-min samples were used to deduce the maximum amount of [3 H]galactose incorporated. The average ratio obtained from eight separate experiments was one *O*-GlcNAc in every 11 MAP2 molecules (Fig. 4*B*). While it is possible that this number reflects the *in vivo* stoichiometry, some *O*-GlcNAc residues may have been cleaved by glycosidase activity during the purification process or may have been inaccessible to the galactosyltransferase. Therefore, this result probably represents a minimal estimate of the *O*-GlcNAc modification of rat brain MAP2.

MAP2 Is Glycosylated on Its Projection Domain—MAP2 is a rod-like molecule composed of a C-terminal binding domain and a 200-kDa N-terminal projection domain that protrudes away from the MT surface. A protease-sensitive region exists between these two domains, and chymotrypsin (26) or thrombin (20) digestion of MAP2 will generate small binding fragments between 28 and 39 kDa and large projection fragments between 140 and 240 kDa (Fig. 5*A*). It has been shown that phosphorylation occurs within both the projection and binding domains (26), and extensive phosphorylation on MAP2 reduces its ability to promote microtubule assembly or to bind to preformed MT (7).

To elucidate if glycosylation may affect MAP2 binding to MT in a similar manner, we examined the location of the glycosylation sites on MAP2. Briefly, rat MAP2 was digested with thrombin, and the various proteolytic fragments were examined either for their MT binding ability or the presence of carbohydrate. Comparison of the results allowed us to determine which binding or projection fragments of MAP2 contained carbohydrate. Galactosyltransferase and the biotin hydrazide/streptavidin system were both used to track the carbohydrate-containing fragments. As expected after limited proteolysis, several high molecular weight projection fragments of MAP2 were generated that lost their MT binding ability and remained in the supernatant, while several low molecular weight binding fragments around 30 kDa cosedimented with MT after incubation with Taxol-stabilized tubulin (Fig. 5*B*). Most of the large projection fragments of MAP2 contained both the [3 H]galactose-labeled *O*-GlcNAc residues and the biotin hydrazide-reactive carbohydrate, while none of the small MT-binding fragments were labeled with either reagent (Fig. 5, compare *B* and

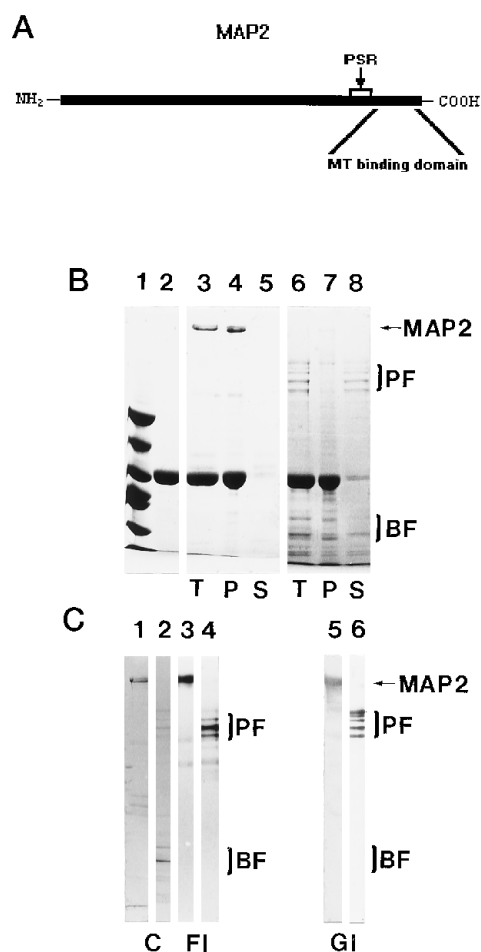


FIG. 5. Localization of carbohydrates on MAP2. Rat MAP2 was incubated with either distilled water or thrombin at 37 °C for 1 h. The samples were subsequently analyzed for their MT binding ability (*B*) or tested for the presence of carbohydrates (*C*). The projection fragments (*PF*) and binding fragments (*BF*) are indicated. *A*, shown is a schematic drawing of MAP2. *PSR*, protease-sensitive region. *B*, Taxol-stabilized tubulin was used to test the MT binding ability of undigested MAP2 (lanes 3–5) or thrombin-digested MAP2 (lanes 6–8). Lane 1, molecular mass markers (97, 66, 55, 42, 40, and 30 kDa); lane 2: Taxol-stabilized tubulin. The whole mixture (*T*), pellet (*P*), and supernatant (*S*) were separated by 4–12% SDS-PAGE and stained with Coomassie Brilliant Blue. *C*, samples were galactosyltransferase-labeled, separated by 4–12% SDS-PAGE, and analyzed by fluorography (*F*) following Coomassie staining (*C*). Alternatively, samples were separated by SDS-PAGE, transferred to nitrocellulose paper, and analyzed by the glycoprotein detection kit (*G*). Lanes 1, 3, and 5, undigested MAP2; lanes 2, 4, and 6, thrombin-digested MAP2. The results indicate that the projection domain of MAP2 contains the carbohydrate moieties. Also note a slight difference in labeling patterns between the [³H]galactose-labeled fragments and the biotin hydrazide-labeled fragments, suggesting that multiple carbohydrate moieties may be present.

C). The largest proteolytic fragment retained MT binding ability and was also glycosylated, presumably containing both the binding domain and a partial projection domain due to incomplete proteolysis. Since carbohydrate was absent from the binding domain, it is unlikely that the carbohydrate directly regulates the MT binding ability of MAP2; instead, the carbohydrate modification might play a role in maintaining MAP2 conformation or mediating its interaction with other cellular components.

MAP2 Contains as Many as Three Sites of O-GlcNAc Modification—Although the average ratio of O-GlcNAc modification on the isolated heat-stable rat brain MAP2 samples was less than one GlcNAc/protein molecule, it was possible that multiple sites on the protein could be O-GlcNAc-modified. To address

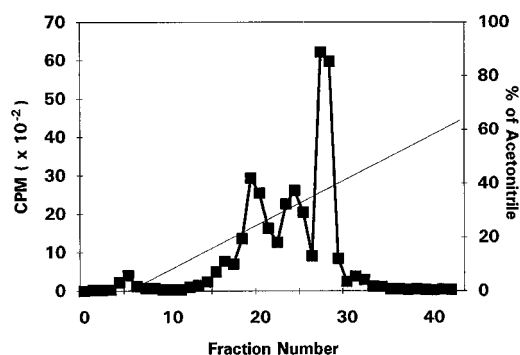


FIG. 6. Tryptic mapping of rat MAP2. Boiled rat brain MAP2 was labeled by [³H]galactose, lyophilized, and digested with trypsin as specified under “Experimental Procedures.” The peptides were then applied to a PeprPC HR 5/5 FPLC column and eluted at a flow rate of 0.5 ml/min. The volume of each fraction is 0.5 ml. 30- μ l solutions from each fraction were analyzed by scintillation counting, and the counts/minute of each fraction were plotted against the elution position and superimposed with the concentration of acetonitrile. Three ³H-containing peaks eluting at ~25, 33, and 38% acetonitrile were obtained.

this possibility, we digested [³H]galactose-labeled MAP2 with trypsin and separated the proteolytic fragments using a C₂/C₁₈ reverse-phase column by FPLC. After extensive digestion of MAP2 by trypsin, the resulting peptides were separated on the reverse-phase column using a linear acetonitrile gradient (see “Experimental Procedures”). The amount of tritium recovered in each fraction was analyzed by scintillation counting. Two independent experiments yielded identical results, and the distribution of radioactivity from one experiment is shown in Fig. 6. Three labeled peaks were observed eluting at 25, 33, and 38% acetonitrile, respectively. The third peak was the most heavily labeled, suggesting several possibilities. First, this peptide may have contained an O-GlcNAc modification site that was more accessible to the galactosyltransferase; second, this site was more commonly modified *in vivo*; or third, more than one O-GlcNAc modification site existed on this peptide. The tryptic mapping results suggest that at least three sites on rat brain MAP2 are capable of being modified by O-GlcNAc. Since the rat brain MAP2 was obtained from the whole brain homogenate, it is possible that different populations of MAP2 molecules exist *in vivo*, some of which are glycosylated while others are not, analogous to phosphorylation.

DISCUSSION

The results presented here demonstrate that high *M_r* MAPs can be considered as glycoproteins as indicated by both their reactivity to biotin hydrazide following periodate oxidation and labeling with [³H]galactose following galactosyltransferase incubation. Using galactosyltransferase, we have shown that brain MAP2 and HeLa MAP4 incorporate [³H]galactose, demonstrating the presence of terminal nonreducing GlcNAc residues. Furthermore, we have shown that the GlcNAc residues on MAP2 are directly O-linked to the protein as a monosaccharide. MAP4 is also modified by single GlcNAc residues as the final β -elimination product of MAP4 eluted as a single peak at the same position as the β -elimination product of MAP2 (data not shown). Our results also suggested that there was an increase in the level of the O-GlcNAc modification in the mitotic MAP4 sample as compared with the interphase sample. This observation is similar to a previous report that showed that keratins 8 and 18 both have an elevated O-GlcNAc modification level in mitotic arrested human epithelial cells (27) and may indicate a cell cycle-dependent regulation of the O-GlcNAc modification on MAP4.

In an effort to ascertain the location of the saccharide moiety associated with the MAPs, we generated fragments of MAP2

following thrombin digestion. The brief thrombin-induced digestion of MAP2 generated a limited number of high molecular weight bands that did not bind to Taxol-stabilized MTs. The complexity of the bands was probably due to incomplete digestion of the MAP2 sample. All the high molecular weight projection fragments could be labeled by both galactosyltransferase and biotin hydrazide; however, the comparative labeling intensity of the individual bands was obviously different. This may simply reflect the inability of galactosyltransferase to access all of the available *O*-GlcNAc residues, or it could indicate that in addition to the simple *O*-linked GlcNAc residues identified by the galactosyltransferase, there are other carbohydrate modifications of the MAP detected by the biotin hydrazide reaction. Tryptic mapping of [³H]galactose-labeled rat brain MAP2 using reverse-phase FPLC showed that three distinct peaks were present. Therefore, at least three different sites on the projection domain of MAP2 may be *O*-GlcNAc-modified. However, confirmation of this result will require identification of the *O*-GlcNAc-modified peptides and sequence analysis.

Despite the presence of multiple modification sites, we obtained results suggesting a low overall stoichiometry of *O*-GlcNAc on MAP2. We believe that this labeling density may be an underestimate of the *in vivo* amount of *O*-GlcNAc on MAP2 for several reasons. First, our detection methods using galactosyltransferase might only label a portion of the total *O*-GlcNAc residues present on the MAP due to inaccessibility of the galactosyltransferase. Second, no precautions were taken to preserve the glycosylation state of the protein during purification, and some of the *O*-GlcNAc residues could have been lost due to the exposure of the protein to glycosidases in the brain extract. Third, there might be different subpopulations of glycosylated MAPs *in vivo*. However, whether such subpopulations of MAP2 exist remains to be determined. It is also possible that a single MAP2 molecule could contain multiple *O*-GlcNAc modification sites *in vivo* as well.

Evidence for glycosylation, both in the form of *O*-GlcNAc and more complex carbohydrate, has also been demonstrated in other proteins associated with the cytoskeleton. Cytokeratins 13 (13) and 8 and 18 (14) have all been shown to contain the *O*-GlcNAc modification. In addition, four keratin isoforms present in human keratinocytes have been demonstrated to be reactive to several monoclonal antibodies raised against keratan sulfate, and immunocytochemical analysis using these keratan sulfate antibodies confirms that keratin filaments are one source of cellular immunoreactivity in these keratinocytes (28). Neurofilament subunits NF-L and NF-M have also been shown to contain *O*-GlcNAc, with stoichiometries similar to those reported here for MAP2 of 0.1 and 0.15 mol of GlcNAc/mol of protein for NF-L and NF-M, respectively (15). It should be noted, however, that the level of modification determined for the neurofilament proteins was only obtained after analysis on Dionex CarboPAC PA1 of the GlcNAc residues released following acid hydrolysis. When measuring the ratio of incorporated galactose to proteins, as we have reported here for MAP2, levels of only 0.011 and 0.028 mol of Gal/mol of protein for NF-L and NF-M, respectively, were obtained (15). Thus, the ratio of modification we obtained for MAP2 should also be considered as the minimal degree of MAP2 modification.

Various studies with *O*-GlcNAc-modified proteins have revealed that the *O*-GlcNAc residues provide different functions in different proteins (9). These functional properties could be dependent on the particular glycosylation site, requiring site-specific transferases analogous to phosphorylation consensus sequences. One of the putative functions of *O*-GlcNAc is to regulate protein phosphorylation. For example, *O*-GlcNAc

modification may modulate phosphorylation by occupying or blocking the phosphorylation site. Mutually exclusive modifications of phosphorylation and glycosylation have been shown in the C terminus of RNA polymerase II; this fragment was either phosphorylated or *O*-GlcNAc-modified, but not both (29). This mutually exclusive model is supported by the notion that the identified *O*-GlcNAc modification sites on many proteins resemble a number of proline-directed kinase sites. Interestingly, the high M_r MAPs contain several consensus proline-directed kinase phosphorylation sites, and MAP4 has been shown to be a substrate for p34^{cdc2} (7). Alternatively, the *O*-GlcNAc modification of one protein could regulate phosphorylation on a second protein, as in the case of *O*-GlcNAc-modified p67. When deglycosylated, p67 loses its ability to protect the eIF-2 α -subunit from phosphorylation (12). *O*-GlcNAc modification does not necessarily inhibit phosphorylation, however, as both phosphorylation and *O*-GlcNAc modification levels on keratins 8 and 18 in the mitotic arrested human colonic cell line HT29 were higher compared with control cells (27). In addition to phosphorylation, it has been hypothesized that *O*-GlcNAc may be crucial for intracellular protein-protein recognition in a manner similar to lectins or in the regulation of a protein's susceptibility to protease (9).

Some or even all of these putative roles for *O*-GlcNAc could be potentially important for MAP2 and MAP4 functions. Both MAP2 and MAP4 have been shown to interact with many cytoplasmic components in addition to MT. With regard to MAP4, a recent report has shown that cyclin B binds to MAP4 (7). Thus, bound cyclin B localizes p34^{cdc2} kinase, a potential regulator of M phase MT dynamics, to the MT. Furthermore, other kinase activities have been found to associate with other MAPs (30). How the binding of these cellular components to MAPs is regulated remains largely undefined. It is known that MAP2 and MAP4 are phosphorylated at multiple sites and in a regulated manner. The MAP phosphorylation state might in turn control the interaction between MAPs and other cellular components. The phosphorylation state of MAPs could in part be regulated by *O*-GlcNAc modification as observed for other *O*-GlcNAc-modified proteins. Alternatively, the *O*-GlcNAc on MAP2 and MAP4 may directly regulate the binding and/or phosphorylation of MAP-associated proteins, such as kinases, that may be crucial for the biological functions of these associated proteins.

From the results that we have obtained, it would appear that glycosylation of the high M_r MAPs is a general post-translational modification of these proteins. Previous studies have provided evidence suggesting that MAPs might be glycosylated *in vivo*. It has been reported that tau protein, a low molecular weight MAP, is present in a glycosylated form in paired helical filaments that are components of neurofibrillary tangles in brain tissue of Alzheimer's disease patients (22, 23). It was also reported that glycation of tau decreased the binding affinity of tau proteins for tubulin, which is another characteristic of tau obtained from Alzheimer's tissue. Claustrin, a chicken brain homologue of MAP1B, has been shown to be sensitive to keratanase treatment, and the protein was reported to be reactive with monoclonal antibodies raised against cartilage keratan sulfate (24). In addition, claustrin was shown to incorporate [³H]glucosamine when added to the culture medium of chick glial cultures, further indicating its glycoprotein nature. Briones and Wiche (31) demonstrated that certain antibodies specific for MAP1 and MAP2 identified a sulfoglycoprotein component of the extracellular matrix secreted by 3T3 cells. While these results were interpreted as cross-reaction of these MAP antibodies with a distinct extracellular glycoprotein, it is also possible that the sulfoglycoprotein antigen detected in the

extracellular matrix was a secreted form of MAP. This would correlate with the hypothesis that the keratan sulfate-containing proteoglycan claustrin might represent a secreted form of MAP1B (24). It has also been suggested that MAP1B may exist as a transmembrane cell-surface protein in rat cortical cell cultures and may be involved in synaptogenesis (32). All these reports indicate the possibility that high M_r MAPs may exist in different populations that may differ in their amino acid composition, post-translational modification (phosphorylation and glycosylation), and/or distribution.

We have also found that high M_r MAPs, such as MAP2 and MAP4, exhibit a high degree of sensitivity to keratanase digestion.² However, our observations indicate that keratanase digestion did not generate a stable core protein for each of the high M_r MAPs. We also observed that the addition of keratan sulfate to the reaction mixture failed to inhibit the keratanase digestion of MAPs. Furthermore, the chemical deglycosylation of MAPs with trifluoromethanesulfonic acid did not induce an observable molecular weight shift in the protein following SDS-PAGE analysis. We were also unable to stain rat brain MAPs with the keratan sulfate-specific antibody 5D4.² Thus, it appears unlikely that MAP2 or MAP4 contains complex carbohydrate modifications, such as has been reported for claustrin. Indeed, if other high M_r MAPs contain complex carbohydrates like keratan sulfate, it would be against prevailing models of glycosylation. Complex glycoproteins generally exist either at the cell surface or within luminal compartments. Nevertheless, several reports have indicated that some glycoproteins are present in the nucleus and cytosol. For example, it has been shown that while chondroitin sulfate proteoglycans are exclusively extracellular in 7-day postnatal brains, they become predominantly cytoplasmic in adult brains (33, 34). The question of how various complex carbohydrate structures have reached the nucleus and/or cytoplasm has been answered in at least one case. A unique type of nuclear heparan sulfate has been shown to be originally synthesized by conventional glycosylation pathways onto heparan sulfate proteoglycans, which are subsequently secreted. After being specifically endocytosed, the free heparan sulfate is released from the proteoglycans, further modified, and transported into the nucleus (34). Tentative complex carbohydrate moieties on high M_r MAPs such as MAP1B could be added via similar pathways. Alternatively, high M_r MAPs could be glycosylated by an unknown cascade of glycosyltransferases in the cytosol. In fact, several glycosyltransferase activities have been demonstrated in the cytosol and nucleus (8).

The biological functions of complex carbohydrates on cytoplasmic and nuclear proteins are poorly understood; however, it has been suggested that they might serve to stabilize proteins in an otherwise unfavorable conformation. High M_r MAPs have long projection domains that are thought to extend away from the surface of the MT. It is possible that carbohydrate moieties associated with the projection domain help maintain this extended conformation. Carbohydrate moieties located

within the projection domain could also prevent untimely proteolysis of this extended region of the protein within the cytoplasm.

In summary, this report has shown for the first time that glycosylation is a common post-translational modification shared by high M_r MAPs. In addition, we have added MAP2 and MAP4 to the growing list of *O*-GlcNAc-modified proteins. We are currently in the process of identifying the sites modified by *O*-linked GlcNAc on MAP2. Further work will be necessary to investigate the keratanase-sensitive feature of the high M_r MAPs and to determine the functional consequences of MAP *O*-glycosylation.

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² M. Ding and D. D. Vandré, unpublished observations.