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Dynamic glycosylation of the microtubule-associated protein tau and neurodegenerative disease.

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DYNAMIC GLYCOSYLATION OF THE MICROTUBULE-ASSOCIATED PROTEIN TAU AND NEURODEGENERATIVE DISEASE

by

CHRISTOPHER SHANE ARNOLD

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
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ABSTRACT OF DISSERTATION
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Title Dynamic Glycosylation of the Microtubule-associated Protein Tau and Neurodegenerative Disease

O-GlcNAcylation of nuclear and cytoplasmic proteins is a highly dynamic post-translational modification consisting of the attachment of a single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) moiety linked to side chain hydroxyls of serine and threonine residues. Tau is a family of phosphoproteins important for regulating microtubule stability and assembly in neurons. In Alzheimer's disease tau is abnormally hyperphosphorylated, no longer binds microtubules, and self-assembles to form paired helical filaments that likely contribute to neuron death. Tau is multiply modified with *O*-GlcNAc, a modification that is analogous with phosphorylation. Bovine tau splicing variants are *O*-GlcNAcylated at over 12 sites, with an average stoichiometry of greater than four moles/mol of tau. Gas-phase sequencing of [^{3}H]galactosylated tau tryptic peptides localized a major attachment site to a domain involved in microtubule binding and containing amino acid Ser-262, a paired helical filament (PHF)-tau phosphorylation site in Alzheimer's disease brain. Glycosylation of this region was verified by *in vitro* *O*-GlcNAcylation of a synthetic peptide by rat brain extract. Manual Edman degradation of a peptide located within the carboxyl-terminal region of tau identified an additional *in vitro* site of *O*-GlcNAcylation as Ser-400, a glycogen synthase kinase 3β (GSK3β) site of phosphorylation and PHF-tau phosphorylation site unique to Alzheimer's brain. Alkaline

phosphatase digestion of *in vitro* transcribed and translated human tau followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and two-dimensional non equilibrium pH gradient gel electrophoresis (2D-NEPHGE) demonstrated that tau is phosphorylated in rabbit reticulocyte lysates. Additionally, a 120-kDa brain-specific polypeptide (p120) immunoreactive to antigen-purified *O*-GlcNAc transferase antibody associated with rat brain microtubules. Electrospray mass spectrometry (ESMS) of tryptic peptides identified p120 as the heavy chain of neuronal-specific kinesin. The *O*-GlcNAcylation of tau, its relationship to tau phosphorylation, and its putative involvement in the etiology of neurodegenerative diseases such as Alzheimer's disease are discussed.

DEDICATION

I dedicate this dissertation to the Honor of that great God and the good of his Church through Jesus Christ our Lord and only Savior.

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LIST OF ABBREVIATIONS

AD, Alzheimer's disease

β-APP, β-amyloid precursor protein

β(1,4)GT, β(1,4)-galactosyltransferase

CaMKII, Ca++/calmodulin-dependent protein kinase II

ConA, concanavalin A

FTDP-17, frontotemporal dementia linked to chromosome 17

GSK3β, glycogen synthase kinase 3β

HSS, high-speed supernatant

IF, intermediate filament

LSS, low-speed supernatant

MAP, microtubule-associated protein

NF, neurofilament

NFT, neurofibrillary tangle

OGT, *O*-GlcNAc transferase

O-GlcNAc, *O*-linked *N*-acetylglucosamine

O-GlcNAcase, *O*-GlcNAc β-D-*N*-acetylhexosaminidase

PHF, paired helical filament

PHF-tau, paired helical filament tau

PKA, cyclic-AMP-dependent protein kinase

PKC, protein kinase C

PMA, phorbol 12-myristate 13-acetate

PP1, protein phosphatase 1

PP2A, protein phosphatase 2A

Pro, proline

PSP, progressive supranuclear palsy

PUGNAc, *O*-(2-acetomido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate

PVDF, polyvinylidine difluoride

RP-HPLC, reverse-phase high performance liquid chromatography

SDS, sodium dodecyl sulfate

Ser, serine

sWGA, succinyl wheat germ agglutinin

TBST, 10 mM Tris, pH 8.0, 100 mM NaCl, and 0.05% Tween-20

Thr, threonine

TPR, tetratricopeptide repeat domain

UDP, uridine diphosphosphate

UDP-GlcNAc, uridine diphospho *N*-acetylglucosamine

Val, valine

WGA, wheat germ agglutinin

INTRODUCTION

Neurodegenerative Diseases and Tau--Alzheimer's disease (AD) and other related neurodegenerative disorders are described by a progressive loss of memory, resulting in dementia and death. They affect more than 20 million people worldwide and their frequency is expected to double over the next 30 years. Therefore, it is crucial to determine the mechanism of nerve cell degeneration affected in these diseases. Currently, the diagnosis of specific dementias is based on clinical assessment and confirmed only post mortem at autopsy. As such, examination of an AD brain reveals the presence of two major pathological lesions, neuritic senile plaques and neurofibrillary tangles (NFTs), originally described by Alois Alzheimer in 1906 (1). Understanding the composition of these pathological lesions will potentially help elucidate the mechanism of their formation and ultimately help define the etiology of neurodegeneration manifested in these disorders.

Biochemical characterization of these lesions has determined that the plaques associated with diseased brains are composed of amyloidogenic peptides generated from the abnormal processing of the β-amyloid precursor protein (β-APP) (2-4). Genetic evidence has demonstrated that mutations in the β-APP which give rise to β-amyloid are associated with a small percentage of familial-associated AD cases (5). Therefore, the abnormal processing of the β-APP has been suggested to play a role in nerve cell death. However, abundant amyloid deposits can be detected in cognitively normal individuals,

but the presence of intracellular NFTs described in these cases demonstrates a stronger correlation with dementias (6, 7).

NFTs are composed primarily of paired helical filaments (PHFs) which form within neurons of the cerebral cortex, the hippocampus, and some subcortical nuclei. Several groups using chemical as well as immunological methods have determined that PHFs are composed primarily of abnormally hyperphosphorylated tau protein (PHF-tau) (8-12). Filamentous pathological tau lesions are central to numerous other neurodegenerative disorders such as Pick's disease, Down's syndrome, progressive supranuclear palsy (PSP), corticobasal degeneration, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (13). The presence of NFTs in several pathologies and its correlation with cognitive impairment indicate that the formation of PHF-tau is likely a central event associated with neuronal death. Deciphering the mechanism of hyperphosphorylated tau formation, therefore, is fundamental to our understanding of the etiology of neurodegenerative disease.

The neuronal cytoskeleton is a composite of structural proteins important for cellular morphology, organelle transport, and the regulation of neurotransmitter release (14, 15). The cytoskeleton consists of microtubules, microfilaments, intermediate filaments, and actin-membrane-bridging proteins. Microtubule networks in the axons of neurons form the framework for the mobilization of vesicles to and from the synaptic termini. Linking and bundling microtubules together are a family of phosphoproteins known as microtubule-associated proteins, (MAPs). Tau, a major MAP in mammalian brain, localizes to microtubule networks in the axon of neuronal cells and influences neurite outgrowth and neuritic stability (16-20) and is therefore thought to be important for proper neuronal cell development (for reviews see Refs. 21-23).

In 1975, Weingarten and others (24) originally described a heat-stable protein they termed tau (τ) that co-purified with microtubules and facilitated the polymerization of tubulin *in vitro*. Thereafter, the microtubule-associated tau proteins were purified to near homogeneity and were demonstrated to migrate as a family of closely related polypeptides from 55-62 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gels (25). The subsequent cloning of tau from mouse (26), human (27), and bovine brains (28) demonstrated that the multiple proteins are generated from the alternative splicing of a single gene. Structurally, isoforms have been determined to consist of an acidic amino-terminal region containing two variably spliced exons, as well as a basic carboxyl-terminal region that is proline rich and contains either three or four repeated microtubule binding sequences (29). The microtubule binding repeats within the carboxyl-termini are 97% conserved among mammals and, together with the adjacent proline-rich sequences. have been implicated in the binding and bundling of microtubules (30-33).

As noted, abnormally hyperphosphorylated tau (PHF-tau) is the major proteinaceous component comprising the pathological NFTs associated with numerous neurodegenerative disorders. PHF-tau is at least partially phosphorylated at 25 sites (34) and has a characteristic stoichiometry of ~6-8 moles PO_4/mol of tau. This hyperphosphorylation occurs at serine (Ser) and threonine (Thr) residues and has been similarly but not identically catalyzed *in vitro* by MAP kinase (erk2)(35), glycogen synthase kinase 3β (GSK3β)(36-39), cdc2 (40), and others (41). PHF-tau self-associates, and its ability to bind polymerized microtubules (42) or promote microtubule assembly (43) is severely compromised. The sites of phosphorylation on PHF-tau identified by mass spectrometry and protein sequencing lie mainly within the proline-rich region amino-terminal to the microtubule tandem binding repeats but have also been identified near the C-terminus

(44). Phosphorylation of the proline-rich region upstream of the tandem repeats and at Ser-262 within the first microtubule binding repeat causes the loss of binding of tau to taxol-stabilized microtubules *in vitro* (32). Restoration of the binding of PHF-tau and tau phosphorylated *in vitro* occurs upon dephosphorylation with protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) (45-48). These data support the hypothesis that potential defects in the regulation of tau phosphorylation cause defects in microtubule binding; abnormal axonal transport; and, ultimately, neuron death.

O-linked N-acetylglucosamine (O-GlcNAc): A Novel Dynamic and Abundant Modification--Numerous nuclear and cytoplasmic proteins are dynamically glycosylated at multiple sites by single *O*-GlcNAc moieties. *O*-GlcNAcylation of proteins occurs at Ser and Thr residues and is present in all higher eukaryotes. Unlike other forms of protein glycosylation, *O*-GlcNAc is a single monosaccharide that turns over much more rapidly than the proteins to which it is attached (for reviews see Refs. 49-53). Characteristically, *O*-GlcNAc is found on phosphoproteins that form multimeric complexes and has the hallmark traits of a regulatory modification. Examples from the rapidly expanding list of proteins modified by *O*-GlcNAc include proteins associated with the nuclear pore complex, transcription factors, cytoskeletal, and membrane-associated proteins. Surprisingly, many sites of *O*-GlcNAc addition are similar to consensus sites of protein phosphorylation (54) and, in some cases, are identical (55, 56). Accordingly, the reciprocal *O*-GlcNAcylation and *O*-phosphorylation, or same-site occupancy, of proteins has been suggested. By occupying the same corresponding hydroxyl of a potential phosphorylation site, *O*-GlcNAc has been suggested to possibly regulate the phosphorylation state of some proteins.

Other similarities between *O*-GlcNAcylation and protein phosphorylation include the regulation of these modifications enzymatically. Like protein kinases and phosphatases, which specifically attach and remove phosphate, an *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase likewise specifically attach and remove *O*-GlcNAc. OGT was originally purified from rat liver (57) and subsequently cloned from rat (58) and *C. elegans* (59). Structurally, OGT is unlike any previously identified glycosyltransferase. Within its amino-terminus are several tandem tetratricopeptide repeat domains (TPRs), regions that are thought to play an important role in mediating intra- and intermolecular protein-protein interactions (60). The TPRs have been demonstrated to regulate exquisitely the enzyme's affinity for uridine diphospho *N*-acetylglucosamine (UDP-GlcNAc) (61), the donor sugar nucleotide in nuclear and cytoplasmic *O*-GlcNAcylation. OGT is also modified by tyrosine phosphorylation and *O*-GlcNAc, two modifications that may additionally alter its activity. Together, protein *O*-GlcNAcylation and protein phosphorylation are analogous dynamic post-translational modifications that appear to have multifaceted functions depending upon the proteins modified. A further detailed description of protein *O*-GlcNAcylation and its regulation, putative functions, and relationship with protein phosphorylation is given in the section entitled, "Dynamic Cytoskeletal Glycosylation and Neurodegenerative Disease."

Hypothesis for Research--Many cytoskeletal proteins such as neurofilaments (62), synapsins (63, 64), and cytokeratins (65), as well as the high molecular weight MAPs (66), have been shown to be modified by *O*-GlcNAc. Also, given phosphorylation's likely role in the formation of PHF-tau and the discovery that many cytoskeletal phosphoproteins are also *O*-GlcNAcylated, tau was probed for this novel modification. This

dissertation discusses the glycosylation of tau by *O*-GlcNAc; the relationship between tau *O*-GlcNAcylation and phosphorylation; and, ultimately, how the *O*-GlcNAcylation of tau may be important in the formation of PHFs.

For instance, PHF-tau isolated from AD brains is phosphorylated on at least 25 sites, and tau is a substrate for numerous protein kinases *in vitro*, including GSK3β, p34cdc2, MAP kinase, and others. The concerted activation of numerous protein kinase pathways would likely be required to achieve the extraordinary phosphorylation state of tau found in PHFs. Regardless, only a subset of the PHF-tau sites are phosphorylated by these kinases *in vitro*. However, due to the facts that glucose utilization is severely impaired in AD brains (67, 68) and that UDP-*N*-acetylhexosamine biosynthesis is sensitive to the availability of glucose (69), a significant reduction in UDP-GlcNAc levels would be expected. This could ultimately lead to a reduction in the overall *O*-GlcNAcylation of proteins in AD brains. Reduced tau *O*-GlcNAcylation might therefore unmask potential phosphorylation sites, resulting in the formation of PHF-tau and the deposition of NFTs.

Some of the work in this manuscript has been previously reported. The first reprint, "Dynamic Cytoskeletal Glycosylation and Neurodegenerative Disease," was published in *Trends in Glycoscience and Glycotechnology* and is a minireview which discusses the important features of protein *O*-GlcNAcylation; the dynamic relationship between *O*-GlcNAcylation and phosphorylation; *O*-GlcNAcylation of the cytoskeleton, and, ultimately, how the *O*-GlcNAcylation of tau may play a role in NFT formation. The second reprint, "The Microtubule-associated Protein Tau is Extensively Modified with *O*-linked *N*-acetylglucosamine," was published in *The Journal of Biological Chemistry* and describes an investigation into the glycosylation of tau using bovine brain as a source.

Following these reprints is a section describing additional significant findings, including a glycosylation-site analysis on tau with synthetic peptides, the finding that tau is phosphorylated in reticulocyte lysates, and the investigation into the existence of a putative brain-specific *O*-GlcNAc transferase. The relevance to glycosylation of various protein substrates and phosphorylation of tau in normal and diseased states are discussed.

DYNAMIC CYTOSKELETAL GLYCOSYLATION AND NEURODEGENERATIVE DISEASE

by

C. SHANE ARNOLD AND GERALD W. HART

Trends in Glycoscience and Glycotechnology (1999) Vol. 11, 355-370

SUMMARY

O-GlcNAcylation of nucleoplasmic and cytoplasmic proteins is a ubiquitous and highly dynamic modification. It entails the attachment of a single *O*-linked *N*-acetyl-glucosamine (*O*-GlcNAc) moiety *O*-glycosidically linked to side-chain hydroxyls of serine (Ser) and threonine (Thr) residues. The rapidly expanding list of *O*-GlcNAcylated proteins includes RNA polymerase II, nuclear pore, heat shock, and tumor suppressor proteins, nuclear oncogenes, and numerous cytoskeletal and membrane-associated proteins. Many sites of *O*-GlcNAc addition are similar to consensus sites of protein phosphorylation, and in some cases identical. Accordingly, *O*-GlcNAcylation and *O*-phosphorylation appear to be reciprocally related on some proteins. All *O*-GlcNAcylated proteins are phosphoproteins which assemble into tightly regulated reversible multiprotein complexes. Several *O*-GlcNAcylated proteins are key components involved in cytoskeletal assembly and organization, and defects in their regulated multimerization are implicated in several neurodegenerative disorders. Thus, abnormal cytoskeletal *O*-GlcNAcylation may promote defects in regulated protein multimerization and potentiate disease.

INTRODUCTION

The serendipitous discovery of *O*-GlcNAc while probing the cell surface of T-lymphocytes with β(1,4)-galactosyltransferase (β(1,4)GT) has dramatically expanded the realm of protein glycosylation. Unlike secretory or lumenal *N*-linked glycoproteins, *O*-GlcNAc is attached to Ser and Thr residues of nuclear and cytoplasmic proteins (for

reviews see Refs. 1-6). The diverse and widespread distribution of *O*-GlcNAcylated proteins is represented by its presence in diverse organisms such as: viruses, fungi, and protozoa. Among the *O*-GlcNAcylated proteins identified to date are a family of dynamic filamentous phosphoproteins which constitute the cytoskeleton. Phosphorylation regulates their interactions within the cell, however, their abnormal hyperphosphorylation leads to the formation of insoluble filamentous aggregates and is associated with the pathology of a number of neurodegenerative disorders. Interestingly, phosphorylation and glycosylation appear to be reciprocally related within the cytoskeleton. In this review the following focal points will be discussed: the dynamic and widespread distribution of *O*-GlcNAc, the enzymatic regulation of protein *O*-GlcNAcylation, (Ser, Thr)-*O*-GlcNAcylation and (Ser,Thr)-*O*-phosphorylation, *O*-GlcNAcylated cytoskeletal proteins, and ultimately how *O*-GlcNAcylation of the cytoskeleton may be important in many neurodegenerative disorders such as Alzheimer's disease.

O-GlcNAc IS DYNAMIC AND ABUNDANT

Unlike the addition of *N*-linked and *O*-linked oligosaccharides, the *O*-GlcNAcylation of proteins is a highly dynamic process (Fig. 1). For instance, in pulse-chase experiments with U373-MG astroglioma cells, 100% of the *O*-linked GlcNAc on αB-crystallins turned over in 20 h while the polypeptide core levels remained unchanged over a 40-h chase (7). Likewise, acid hydrolysis of keratins (K8, K18) purified from [^{3}H]glucosamine-labeled HT29 human colonic cells revealed that both the biosynthesis and degradation rates of *O*-GlcNAc were faster than the polypeptide (8). *O*-GlcNAc levels

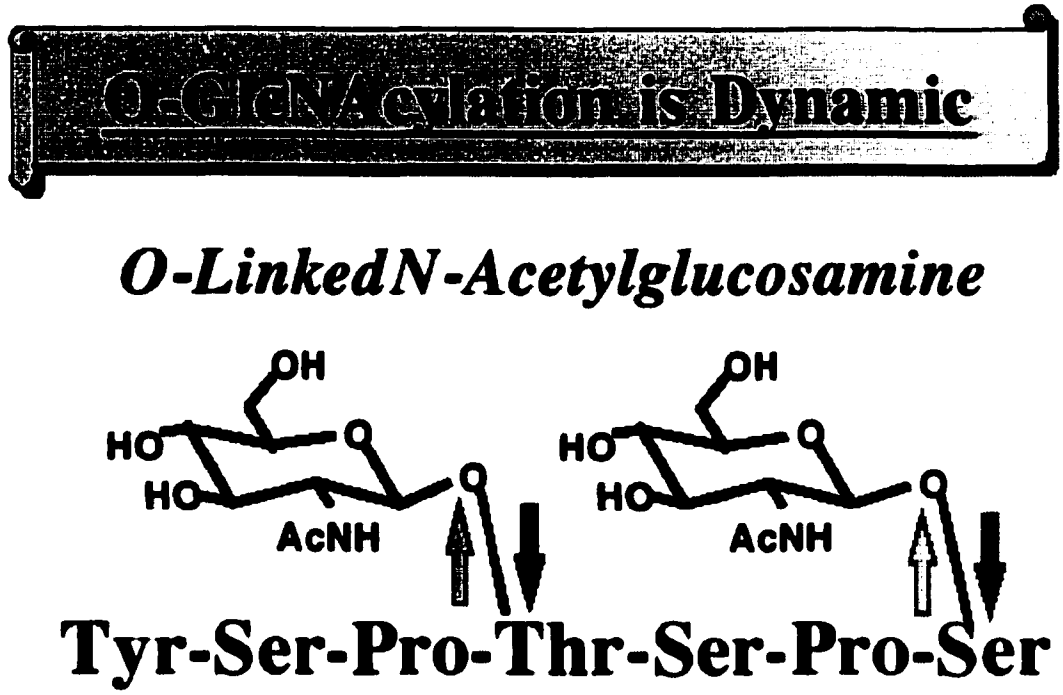

Fig. 1. **The dynamics of protein *O*-GlcNAcylation.** The *O*-GlcNAc modification occurs on serine and threonine residues, and turns over more rapidly than the protein backbone.

also change in response to various cellular stimuli. Kearse and Hart observed a rapid reduction in the *O*-GlcNAcylation of cytoplasmic proteins released from digitonin permeabilized T-lymphocytes when stimulated with Concanavalin A (Con A) (9). Additionally, *O*-GlcNAcylation of nuclear proteins rapidly increased in T cell hybridomas stimulated with Phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore, ionomyocin. Furthermore, treatment of HT29 cells with *O*-(2 -acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), a potent inhibitor of the enzyme responsible for the removal of *O*-GlcNAc, caused an approximate two fold increase in *O*-GlcNAc levels (10). Changes in the *O*-GlcNAcylation of proteins also occur during the cell cycle. For example, the amount of *O*-GlcNAc on human keratin K18 increases seven fold in mitotically arrested HT29 cells, whereas during S-phase, its glycosylation is unchanged (11). Collectively, these data clearly illustrate the dynamics of *O*-GlcNAcylation, and establish this as a unique form of protein glycosylation. In addition, this evidence suggests that *O*-GlcNAc is a regulatory modification.

The number of proteins known to be modified with *O*-GlcNAc is continuously growing. The majority of eukaryotic *O*-GlcNAcylated proteins reside within the nucleus. In fact, approximately 54% of *O*-linked GlcNAc (pmol/mg) is in the nucleus of an average liver cell (12). RNA polymerase II and its associated transcription factors are a few of the many nuclear glycosylated proteins (13). Nuclear pore proteins, including the heavily glycosylated nucleoporin p62, are also modified with *O*-GlcNAc (14, 15). Moreover, the tumor suppressor p53 (16), oncoproteins c-Myc (17) and v-Erb-a (18), as well as the estrogen receptor (19) and SV40 large T antigen (20), are all *O*-GlcNAcylated.

In addition to nuclear proteins, *O*-GlcNAcylation also occurs on cytoskeletal, cytosolic, and membranous proteins throughout the cytoplasm. Several *O*-GlcNAcylated proteins have been identified within the cytoskeleton (Fig. 2), including a dynamic network of intracellular proteins involved in various functions from muscle contraction, axonal transport, to neuromuscular junction formation. Microtubule-associated proteins (MAPs), homologous phosphoproteins (21-23) which facilitate the polymerization of tubulin (24), are among the many *O*-GlcNAcylated proteins which constitute the cytoskeleton. The high molecular weight MAPs: MAP1, MAP2, and MAP4 (25), as well as the lower molecular weight family of tau proteins (26), are all modified with *O*-GlcNAc. The intermediate filament keratins, K13, K8, and K18 which are involved in maintaining the structural integrity of cells, are also *O*-GlcNAcylated. The neurofilaments (NFs) NF-L, NF-M, and NF-H which are important in nerve conduction velocity and implicated in motor neuron diseases, likewise are *O*-GlcNAcylated (27, 28). In addition, ankyrins which are targeted to Nodes of Ranvier, specific domains within myelinated axons, are modified with *O*-GlcNAc (29). Other glycosylated cytoskeletal proteins include the αA- and αB-crystallins (7, 30), the erythrocyte membrane protein Band 4.1 (31), talin (32), vinculin (33), synapsin I (34), and clathrin assembly protein 3 (AP3) (35-37). Cumulatively, it is clearly evident that there are numerous nuclear and cytoskeletal proteins modified with this dynamic post-translational modification. Further investigating these proteins will ultimately reveal the many multi-functional roles of *O*-GlcNAc.

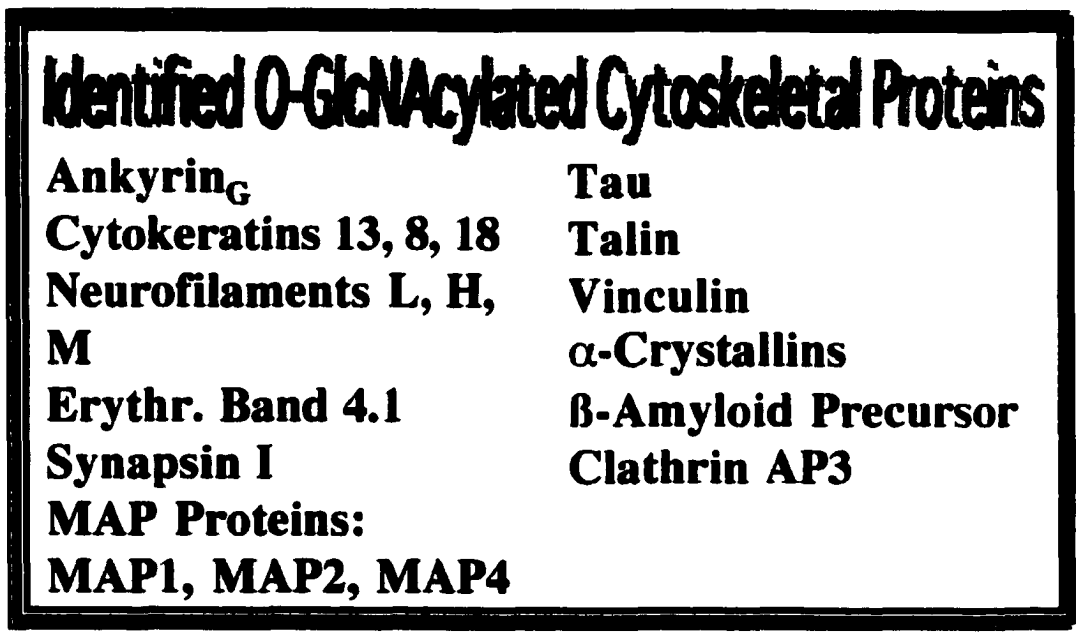

Fig. 2. **A list of some *O*-GlcNAcylated proteins found within the cytoskeleton.** Among them are intermediate filaments, microtubule-associated proteins, and actin-membrane bridging proteins.

THE ENZYMATIC REGULATION OF *O*-GlcNAc

Studying the metabolism of *O*-GlcNAc first requires an identification and understanding of the enzymes involved. Two enzymatic activites, a uridine diphosphate (UDP)-GlcNAc: polypeptide *N*-acetylglucosaminyl-transferase (*O*-GlcNAc transferase, or OGT) and an *O*-GlcNAc specific β-D-*N*-acetylglucosaminidase (*O*-GlcNAcase) are responsible for the addition and removal of *O*-GlcNAc to and from nuclear and cytoplasmic proteins, respectively. OGT was originally purified from rat liver (38), and subsequently cloned from a rat liver cDNA library (39) and from *C. elegans* (40). *O*-GlcNAcase has also been recently purified and characterized from rat spleen cytosol (41). Due to the highly dynamic nature of this modification, both enzymes regulating *O*-GlcNAcylation must be tightly controlled, and therefore thoroughly characterized.

The OGT was originally purified to apparent homogeneity from rat liver cytosol, and is a heterotrimeric complex containing one 78-kDa (β) and two catalytic 110-kDa (α) subunits. Both of the enzyme's subunits are *O*-GlcNAcylated as well as tyrosine phosphorylated. The enzyme has an unusual high affinity for the donor substrate UDP-GlcNAc (K_m = 545 nM), and appears to have multiple affinities for UDP-GlcNAc across a broad physiological range (42) which gives it a competitive advantage over lumenal UDP-GlcNAc transporters. OGT is inhibited by low micromolar concentrations of UDP, UTP, and UDP-GlcNAc. The enzyme has recently been cloned from *C. elegans*, human and rat liver cDNA libraries, and is evolutionarily conserved. However, at the polypeptide level it is uniquely unlike any other known glycosyltransferase. OGT

contains an uncharacteristically high number of tetratricopeptide repeats (TPR), a structural motif implicated in both intra- and intermolecular protein-protein interactions (43). Consequently, the regulation of the enzyme's substrate specificity may depend upon its TPR-associated factors. OGT message, protein, and activity are present in numerous tissues including: brain, heart, kidney, liver, lung, spleen, pancreas and thymus. Among these, OGT is enriched in brain, thymus, and the pancreas.

The enzyme involved in *O*-GlcNAc removal, *O*-GlcNAcase, has been purified over 20,000-fold from rat spleen cytosol. *O*-GlcNAcase is a heterodimeric enzyme consisting of a 54-kDa (α) subunit and a 51-kDa (β) subunit. Unlike other hexosaminidases, this β-D-*N*-acetylglucosaminidase (*O*-GlcNAcase) has a neutral pH optimum (pH 6.4), and is uninhibited by GalNAc or its analogs. *O*-GlcNAcase is however effectively inhibited by GlcNAc (K_i = 3.9 mM) and its analogs 1-azido-GlcNAc (K_i = 1.14 mM), 1-amino-GlcNAc (K_i = 151 μM), and PUGNAc (K_i = 52 nM). *O*-GlcNAcase activity is present in many rat tissues including: liver, kidney, heart, lung, uterus, and spinal cord, but the enzyme is most enriched in the spleen and brain. The cloning of the rat cytosolic enzyme is in progress.

(SER, THR)-*O*-GlcNAcYLATION AND (SER, THR)-*O*-PHOSPHORYLATION

Analogous to (Ser/Thr) protein kinases and protein phosphatases which modulate the phosphorylation state of proteins, OGT and *O*-GlcNAcase regulate the dynamic *O*-GlcNAcylation of proteins (Fig. 3). A large number of *O*-GlcNAcylation sites resemble

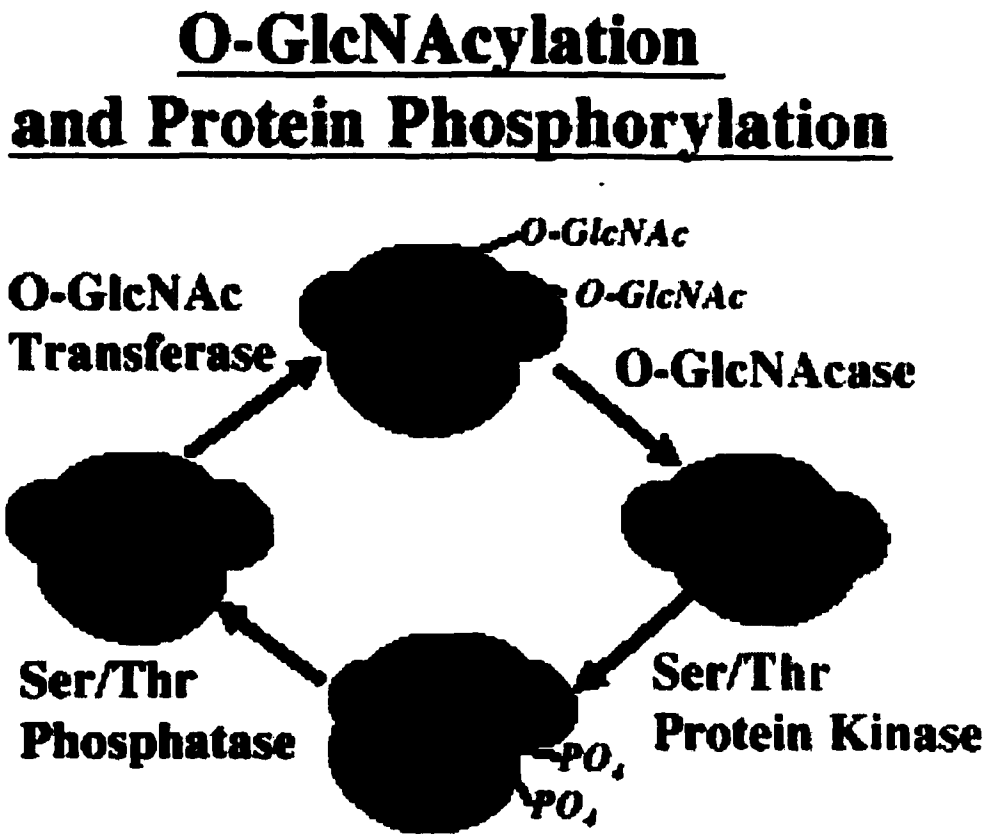

Fig. 3. **Protein *O*-GlcNAcylation and *O*-phosphorylation.** A model representing the multiple pathways involved in regulating the post-translational state of *O*-GlcNAcylated phosphoproteins. Analogous to Ser/Thr protein kinases and phosphatases which attach and remove phosphates, are the *O*-GlcNAc transferase and *O*-GlcNAcase which likewise attach and remove *O*-GlcNAc.

proline-directed (Ser/Thr) protein kinase sites of phosphorylation (5), and lie at or near Pro-Val-(Ser/Thr) sequences. These include sites from the human serum response factor (44), αA-and αB-crystallins (30, 7), and from the head domains of rat NFs NF-L and NF-M (27). Notably, other *O*-GlcNAcylation sites appear to have no definitive identifiable consensus sequence (5).

Because *O*-GlcNAc levels change rapidly upon stimulation with mitogens as well as during the cell cycle, protein *O*-GlcNAcylation has been proposed to be analogous to protein phosphorylation. For instance, both *O*-GlcNAcylation and phosphorylation of human keratins (K8, K18) increase in G_2/M arrested HT29 cells (11, 45). *O*-GlcNAcylation has also been proposed to regulate the phosphorylation state of proteins by steric hinderance or by blocking access to nearby hydroxyl groups. For example, the glycosylation sites of synapsin I, a synaptic-vesicle-associated protein, lie within domains involved in actin binding and closely bracket neighboring Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) sites of phosphorylation (34). Likewise, *O*-GlcNAc sites overlap phosphorylation sites within the head domains of NFs NF-M, and NF-H. The NF NF-H is also extensively *O*-GlcNAcylated in a region consisting of 52 Lys-Ser-Pro repeats, a region that is also highly phosphorylated. Although, it is not clear if these forms of NF NF-H molecules, *O*-GlcNAcylated and phosphorylated, exist as two distinct subpopulations. The close proximity of GlcNAc moieties to nearby sites of phosphorylation hints at a novel mechanism by which the overall post-translational state of these proteins might be regulated.

Due to the fact that many sites of *O*-GlcNAcylation are similar to MAP kinase and glycogen synthase kinase 3β (GSK3β) sites of phosphorylation, and in some cases identical, these two modifications have also been suggested to have a reciprocal relationship. For example, the major site of *O*-GlcNAcylation on the oncogenic transcription factor c-Myc is within its transactivation domain (17) at Thr-58 (46), a known *in vivo* phosphorylation site and the major frequent mutational "hot-spot" associated with tumorigenic lymphomas. Consistent with the hypothesis of *O*-GlcNAcylation and *O*-phosphorylation reciprocity, mutually exclusive subpopulations of *O*-GlcNAcylated and *O*-phosphorylated forms of RNA polymerase II have been isolated from calf thymus (13). Furthermore, increased *O*-GlcNAcylation of the transcription factor Sp1 by treatment of HT29 cells with PUGNAc, resulted in a concomitant marked reduction in Sp1 phosphorylation (10).

Investigators have recently addressed the question of *O*-GlcNAcylation and phosphorylation reciprocity by testing the effects of both protein kinase inhibitors and activators on protein *O*-GlcNAcylation in cerebellar neurons (47). After drug treatments, cells were fractionated into a membrane and cytosolic fraction, and a detergent insoluble cytoskeletal fraction. When cerebellar neurons were treated with either PMA, a protein kinase C (PKC) activator, or dibutyryl cAMP, a cyclic-AMP dependent protein kinase (PKA) activator, protein *O*-GlcNAcylation within the cytoskeletal fraction decreased by 50-60%. Conversely, inhibition of PKC with bis-indoylmaleimide produced a 45% increase in cytoskeletal *O*-GlcNAcylation, and PKA inhibition doubled the amount of *O*-GlcNAc detected in the cytoskeletal fraction. Consequently, protein phosphorylation

and *O*-GlcNAcylation appear to be reciprocally related within the cytoskeleton. Surprisingly, treatment with these compounds appeared to have little effect on cytosolic or membrane *O*-GlcNAcylation in these studies. Clearly, the complexity of the relationship between these two post-translational modifications is dependant upon which proteins are modified as well as which (*O*-GlcNAcylation/phosphorylation) pathways are either suppressed or activated.

THE O-GlcNAcYLATED CYTOSKELETON

The neuronal cytoskeleton is a composition of dynamic structural proteins important for cellular morphogenesis, organelle transport, and the regulation of neurotransmitter release (22, 23). Cytoskeletal elements also establish neuronal polarity and direct axoplasmic flow of synaptic vesicles to and from the synapse. The cytoskeleton consists of microtubules, intermediate filaments (IFs) including NFs, microfilaments, and actin/membrane bridging proteins. Microtubules (24 nm) are polymers of α, β-heterodimer subunits of tubulin, serve as rails for vesicle transport, and are required for the proper positioning of the mitotic spindle and nucleus in many cell types. Linking and bundling microtubules together are a family of proteins known as MAPs. MAPs co-purify with microtubules and also facilitate the polymer-ization of tubulin *in vitro* (24). The major MAPs in the brain include: MAP1, MAP2, and tau, all of which are multiply *O*-GlcNAcylated. The higher molecular weight MAPs including MAP1, MAP2, and MAP4 have approximate molecular weights of 350, 280, and (200-240) kD respectively. MAP1 and MAP2 are exclusively found in neuronal tissue, whereas MAP4 is ubiquitously expressed

and localizes to microtubules of mitotic and interphase cells. MAP2 is found predominately in dendrites, whereas MAP1 is localized in the cell body, dendrites, and axons of neurons.

Glycosylation of MAPS--MAP1, MAP2, and MAP4 were all demonstrated to be glycosylated by the established method of periodate oxidation followed by detection with biotin hydrazide-streptavidin (25). *O*-GlcNAc was detected on MAP2 by probing for terminal GlcNAc residues with β(1,4)GT and [^{3}H]-UDP-Gal followed by gel filtration analysis of the β-eliminated radiolabeled sugars. Reverse-phase high pressure liquid chromatography (RP-HPLC) of radiolabeled MAP2 digested with trypsin revealed three distinct peaks indicating multiple sites of *O*-GlcNAcylation. Further digestion of the polypeptide with thrombin indicated the presence of *O*-GlcNAc within the projection domain, a region which blocks abnormal MAP2 axonal transit and is required for proper dendritic targeting (48).

The lower molecular weight MAPs consist of a family of phosphoproteins known as tau which have molecular weights (55-65-kDa) and are generated from the alternative splicing of a single gene (49). Tau regulates tubulin bundling and assembly, and stabilizes the microtubule network in the axons of neurons (50, 51). The phosphorylation of tau regulates its metabolism (52-54), its ability to bind and assemble microtubules (55, 56), and its tendency to self-associate (57). With some 17 (Ser/Thr)-Pro regions, tau is a substrate for numerous protein kinases *in vitro* including: GSK3-β (58, 59), MAP kinase

(erk2) (60), a neuronal cdc2-like protein kinase (61), CaMKII (62), casein kinase II (63), PKA (64, 65), and others (66, 67). However, it is still not clear which protein kinases are responsible for the regulation of tau phosphorylation *in vivo*.

O-GlcNAcylation of the low molecular weight family of tau proteins was detected both by blotting with succinylated wheat germ agglutinin (sWGA), and by enzymatically probing with β(1,4)GT and [^{3}H]-UDP-Gal. Structural analysis of the β-eliminated material was confirmed by high pH anion exchange chromatography. All tau splice variants are *O*-GlcNAcylated with an average stoichiometry of ~4 mol GlcNAc/mol protein, similar to the stoichiometry of tau phosphorylation. Tau is *O*-GlcNAcylated at 12 or more sites, suggesting substoichiometric site occupancy. It is unclear what role *O*-GlcNAcylation may have in tau function, but there are a host of possibilities. For instance, *O*-GlcNAc may modulate the phosphorylation state of tau by steric hinderance or direct reciprocity, which in turn would ultimately regulate all of the functions influenced by tau phosphorylation. These might include: regulating the binding and bundling of microtubules, controlling tau degradation, or even governing the localization of tau.

Glycosylation of IFs--Another family of proteins which contribute to the framework of the cytoskeleton are the IFs. IFs are highly dynamic structures composed of interwoven α-helical polypeptides that form coiled-coil dimer subunits creating an array of 10-nm filaments (68). There are five classes of IF proteins. IFs of class I and II are keratins which are broadly expressed in epithelial cells. Keratins K8, K13, and K18 give

mechanical integrity to the stratified squamous epithelium. Single layer squamous epithelia predominately express K8 and K18 as obligate noncovalent heteropolymers (K8/K18), whereas K13 is found mostly in esophageal epithelia (69). Several post-translational modifications have been described for keratins including phosphorylation (70), acetylation (71), and *O*-GlcNAcylation (11).

O-GlcNAc was first detected on keratins (K8/K18) by the established method of probing for terminal GlcNAc residues with β(1,4)GT in the presence of [^{3}H]-UDP-Gal, and analyzing the structure of the labeled sugars by β-elimination and thin-layer chromatography. Keratins (K8/K18) were demonstrated to contain ~(1.5-2) mol *O*-linked GlcNAc/mol protein by chemical analysis. After digesting radiolabeled K8 and K18 with trypsin and elastase respectively, peptide mapping demonstrated the presence of multiple glycopeptides. As described previously, *O*-GlcNAc turnover on keratins (K8/K18) was shown to be faster than polypeptide turnover. Additionally, cell cycle arrest caused a dramatic increase in the *O*-GlcNAcylation of Thr residues on K18, and an increase in the Ser phosphorylation of K8, the obligate partner of K18 in filament assembly. Simultaneously with IF reorganization, keratin *O*-GlcNAcylation increased within head and proximal rod domains. However, mutational analysis suggests that *O*-GlcNAcylation is not required for keratin filament assembly (72). *O*-GlcNAcylation of keratins might nonetheless regulate keratin degradation, localization, or mediate IF-protein interactions.

Class IV IF proteins include NFs NF-L, NF-H, and NF-M, the most abundant structural components in large myelinated axons. NFs assemble as obligate heteropoly-

mers of NF-L (62 kDa), and either NF-H (115 kDa), or NF-M (100 kDa), and are essential for the development of axon diameter, a crucial factor in the velocity of nerve conduction (73). Each polypeptide shares a 310 amino acid α-helical rod domain essential for the assembly of normal filaments, as well as a globular head domain, and variable length tail domain. Regions within both the NH_2-terminal head and carboxyl-terminal domains are required for the proper networking of NFs. All NFs are post-translationally modified by phosphorylation. A majority of the sites of phosphorylation are located within the C-terminal domains, albeit a small percentage also exist within the NH_2-terminal head domains. Phosphorylation within the head domain of NFs has been demonstrated to induce the disassembly of filaments (74), as well as inhibit filament assembly (75, 76). NFs are also *O*-GlcNAcylated within the head domains adjacent to sites of NF phosphorylation.

The *O*-GlcNAcylation of NFs NF-H, NF-L, and NF-M purified from rat spinal cord was first identified by probing for terminal GlcNAc residues with β(1,4)GT in the presence of [^{3}H]-UDP-Gal, and analyzing the structure of the β-eliminated radiolabeled sugars by high pH anion exchange chromatography. Purification of glycopeptides after pro-teolysis identified four major sites of glycosylation in the head domain of NF-L: Thr-21, Ser-27, Ser-34, and Ser-48. Three out of four *O*-GlcNAcylation sites on NF-M were found in the head domain including Thr-19, Ser-34, and Thr-48, whereas the other, Thr-431, is located in the tail region. The *O*-GlcNAcylation of NF-H likewise contains three sites of *O*-GlcNAc attachment in the head domain of the protein, including: Thr-53, Ser-

54, and Ser-56. In addition, NF-H is multiply *O*-GlcNAcylated in the rod domain, a region consisting of 52 Lys-Ser-Pro repeats known to be highly phosphorylated. In accord with mutational studies, the presence of *O*-GlcNAc within the head domains has been proposed to regulate proper filament assembly. For instance, deletion of amino acids 18-30 from NF-L, including both *O*-GlcNAcylation sites Thr-21 and Thr-27, caused a disruption of wild-type NF formation in transfected cells (77). Moreover, NH_2-terminal deletion through the first site of *O*-GlcNAcylation on NF-M caused perinuclear aggregation instead of a proper formation of filament networks (78).

O-GlcNAcylation of Actin-membrane Bridging Proteins--Another collection of *O*-GlcNAcylated cytoskeletal proteins tether the plasma membrane to the underlying network of spectrin-actin filaments. One of the first *O*-GlcNAcylated proteins identified, erythrocyte protein Band 4.1, or protein 4.1, is a member of this family. Band 4.1 is an 80-kDa protein required for the formation of a spectrin-actin-4.1 ternary complex essential for the maintenance of proper red blood cell shape. *O*-GlcNAcylation of Band 4.1 has been demonstrated by β(1,4)GT labeling erythrocyte ghosts in the presence of [^{3}H]-UDP-Gal. Chemical cleavage and digestion with α-chymotrypsin has revealed that Band 4.1 is glycosylated within the carboxyl-terminal 117 amino acids. Interestingly, mutations in this C-terminal region of the molecule, the actin-spectrin binding domain (79), can cause hereditary elliptocytosis resulting in hemolytic anemia (80). Moreover, Band 4.1 knock-out mice have neurological defects in movement, coordination, balance and

learning, in addition to hematological abnormalities (81). Related to Band 4.1 is a 215-kDa cyto-skeletal protein, talin, expressed in many different tissues. Talin is targeted to focal contacts (82), the postjunctional membrane of neuromuscular junctions (83), and interacts with vinculin and the cytoplasmic face of integrins (84). Talin purified from chicken gizzard and porcine stomach is modified with *O*-GlcNAc (32). Two glycosylation sites were identified and are located in the tail domain. *O*-GlcNAcylation of this region may play a role in regulating talin's interaction with vinculin, another *O*-GlcNAcylated protein, or other cytoskeletal proteins.

Recently, ankyrin$_G$ was also identified as another *O*-GlcNAcylated protein which bridges the membrane to the spectrin-actin network. Ankyrin$_G$ is targeted to Nodes of Ranvier, regions within myelinated axons where ion fluxes of action potentials are propagated (85). Ankyrin$_G$ is essential for the proper localization of voltage-dependent sodium channels, and for normal firing in Purkinje neurons (86). Ankyrins also bind the cytoplasmic domains of a family of immunoglobulin/fibronectin type III cell adhesion molecules including: neurofascin, NrCAM, L1, and NgCAM (87). Due to the presence of a 40-kDa Ser/Thr rich domain unique to ankyrin$_G$ (85), its glycosylation was investigated.

480 kDa ankyrin$_G$ extracted from brain membranes bound WGA affinity columns, was labeled by β(1,4)GT and [^{3}H]-UDP-Gal, and cross-reacted with an *O*-GlcNAc-specific antibody (29). The nodal mucin-like Ser/Thr rich domain was determined to be *O*-GlcNAcylated, and mutational analysis indicated this region contributes to the prevention of the lateral diffusion of ankyrin-neurofascin complexes (88). Additionally,

cerebellar ankyrin$_G$ knockout mice develop ataxia, sodium channel mistargeting and progressive Purkinje neuron degeneration (86). Thus, *O*-GlcNAc may modulate the proper development and arrangement of protein-ankyrin complexes at the Nodes of Ranvier required for normal firing in Purkinje cells.

Nerve terminals also contain cytoskeletal membrane bridging proteins. There exists a mechanism that requires vesicle targeting and membrane fusion within neurons for the proficient uptake and release of neurotransmitters. This process of synaptic transmission involves the rapid recycling of synaptic vesicles at the synapse. Concentrated within the nerve terminal are cytoskeleton bridging proteins known as synapsins. Synapsin I sequesters fusion competent vesicles by anchoring them to the proximal cytoskeletal network in a phosphorylation dependent manner (89). Synapsin I is also multiply *O*-GlcNAcylated near neighboring phosphorylation sites within domains dedicated to binding synaptic vesicles (90), and domains required for binding (91), and nucleating (92) actin. Seven *O*-GlcNAcylation sites have been identified on synapsin I using a combination of RP-HPLC, electrospray mass spectrometry (ES-MS), and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (34). The localization of *O*-GlcNAc residues within these domains suggests that *O*-GlcNAcylation of synapsin I may modulate directly, or by regulating synapsin I phosphorylation, its ability to bind and anchor synaptic vesicles to the actin cytoskeleton.

The mechanism of synaptic vesicle reuptake is considered similar to clathrin-coated pit mediated endocytosis, an internalization process common to all cells. Important for the assembly of clathrin-coated vesicles (93), as well as for synaptic vesicle

recycling (94, 95), is the 160-kDa clathrin-assembly protein AP3. *O*-GlcNAcylation of AP3 was demonstrated when peptide *N*-glycosidase F (P*N*Gase F) treated human brain homogenates were probed with β(1,4)GT and [^{3}H]-UDP-Gal, and a radiolabeled 160-kDa AP3 immunoreactive band with the appropriate corresponding isoelectric point was identified (36). Since bacterially expressed AP3 is capable of clathrin binding and assembly (96), and *in vitro* galactosylated AP3 is clathrin assembly competent (97), it is unclear how *O*-GlcNAc affects the function of AP3. However, since both Synapsin I and clathrin AP3 are enriched at the synapse, *O*-GlcNAcylation may play a role in maintaining their localization. *O*-GlcNAc might also regulate the dynamic recycling of vesicles to and from the membrane.

NEURODEGENERATIVE DISEASES INVOLVING O-GlcNAcYLATED CYTOSKELETAL PROTEINS

Neurodegenerative diseases encompass a large number of disorders. Yet, many have a common etiology that is characterized by the formation of filamentous lesions composed of highly phosphorylated cytoskeletal proteins. Alzheimer's disease (AD), for example, is a progressive neurodegenerative disease which has a distinct pathology (98). Two neuropathological hallmarks found in AD brains are neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs) (99), and amyloid plaques composed of peptides generated from the abnormal processing of the β-amyloid precursor protein (β-APP) (100-102). Biochemical characterization of PHFs has determined that they are composed primarily of abnormally hyperphosphorylated tau protein (PHF-Tau)

(103-106). NFTs are also found in a multitude of other neurodegenerative disorders including: frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, progressive supranuclear palsy (PSP), and others (107) (Fig. 4). The presence of NFTs in many pathologies, and its correlation with cognitive impairment (108, 109), indicate that the abnormal hyperphosphorylation of tau is a central event in neuron death. Deciphering the mechanism of hyperphosphorylated tau formation, therefore, is crucial to our understanding of the etiology of neurodegeneration.

Since their enzymatic activities are diminished in Alzheimer brains (110), a possible mechanism may include alterations in protein phosphatases 2A (PP2A) and 2B (PP2B) activities toward tau. Another scenario could include the activation of protein kinase pathways. PHF-Tau isolated from AD brains is phosphorylated on at least 25 sites (111). Tau is a substrate for protein kinases *in vitro* including: GSK3β, p34cdc2, CaM-KII, PKA, MAP kinase, and numerous others. The concerted activation of multiple phosphorylation pathways would likely be required in order to achieve the extraordinary phosphorylation state of tau found in NFTs. Regardless, these kinases phosphorylate a majority, but not all of the sites identified in tau from AD brain (112, 113). However, due to the fact glucose metabolism is significantly impaired in Alzheimer brains (114, 115), and UDP-*N*-acetylhexosamine biosynthesis is sensitive to the availability of glucose (116), a drastic reduction in UDP-GlcNAc levels would be eminent. This could ultimately reduce the *O*-GlcNAcylation of proteins AD brain. Such an observation has already been documented for the cytoskeletal protein AP3 (36, 37). Reduced tau *O*-

Neurodegenerative Diseases with Abundant Tau Filamentous Lesions

Alzheimer's disease
Down's syndrome
Corticobasal Degeneration
Dementia pugilistica
Dementia with tangles
Frontotemporal dementias
Parkinsonism linked to chrom. 17
Myotonic dystrophy
Niemann-Pick disease type C
Parkinsonism-dementia complex of Guam
Pick's disease
Postencephalitic Parkinsonism
Prion diseases with tangles
Progressive supranuclear palsy
Subacute sclerosing panencephalitis

Fig. 4. **Examples of the numerous neurodegenerative diseases in which the MAP-tau is involved.**

GlcNAcylation might therefore ultimately unmask potential phosphorylation sites unique to AD brain and result in NFT formation (Fig. 5).

SUMMARY

The nervous system is a rich source of filamentous proteins critical in maintaining proper neuronal morphology and function. Numerous cytoskeletal proteins have been shown to be modified by *O*-GlcNAc and are involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's. The highly phosphorylated forms tend to precipitate as insoluble filamentous inclusions (NFTs) which leads to blocked axonal transport, inhibition of neurotransmitter uptake and release, and ultimately massive neuron death. The reciprocal relationship between *O*-GlcNAcylation, and *O*-phosphorylation may ultimately be a causative link in the mechanism of pathogenesis for several neurodegenerative disorders.

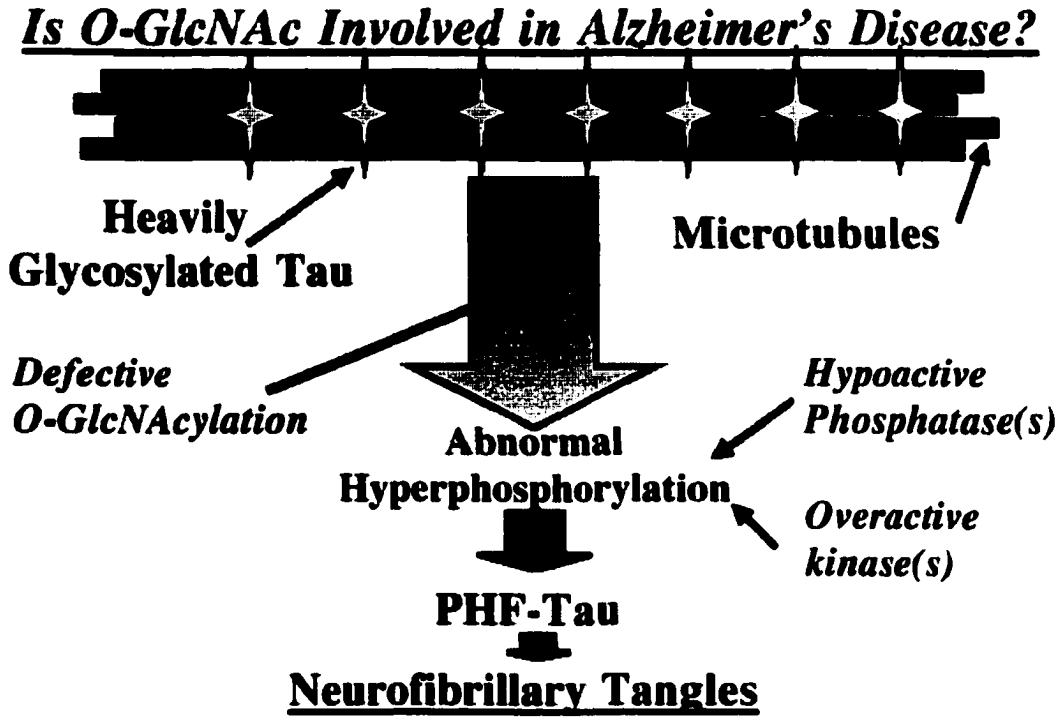

Fig. 5. **Tau *O*-GlcNAcylation and Alzheimer's disease.** A model depicting the outcome of defective tau *O*-GlcNAcylation on neurofibrillary tangle formation, and ultimately neuron death.

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THE MICROTUBULE-ASSOCIATED PROTEIN TAU IS EXTENSIVELY MODIFIED BY *O*-LINKED *N*-ACETYLGLUCOSAMINE

by

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SUMMARY

Tau is a family of phosphoproteins that are important in modulating microtubule stability in neurons. In Alzheimer's disease tau is abnormally hyperphosphorylated, no longer binds microtubules, and self-assembles to form paired helical filaments (PHFs) that likely contribute to neuron death. Here we demonstrate that normal bovine tau is multiply modified by Ser(Thr)-*O*-linked *N*-acetylglucosamine (*O*-GlcNAc), a dynamic and abundant post-translational modification that is often reciprocal to Ser(Thr)-phosphorylation. *O*-GlcNAcylation of tau was demonstrated by blotting with succinylated wheat germ agglutinin and by probing with bovine milk β(1,4)galactosyltransferase. Structural analyses confirm the linkage and the saccharide structure. Tau splicing variants are multiply *O*-GlcNAcylated at similar sites, with an average stoichiometry of greater than 4 mol of *O*-GlcNAc/mol of tau. However, the number of sites occupied appears to be greater than 12, suggesting substoichiometric occupancy at any given site. A similar relationship between average stoichiometry and site-occupancy has also been described for the phosphorylation of tau. Site-specific or stoichiometric changes in *O*-GlcNAcylation may not only modulate tau function but may also play a role in the formation of PHFs.

INTRODUCTION

Tau is a group of closely related MAPs that constitute an important component of the neuronal cytoskeleton (1). Tau proteins are generated by differential mRNA splicing of a single gene resulting in multiple isoforms in the adult mammalian brain (2-4).

Tau is a phosphoprotein (5-7), and this post-translational modification clearly regulates both its function and metabolism (8-10). Hyperphosphorylated tau is the primary component of the paired helical filaments (PHFs) observed in Alzheimer's disease brains (11, 12). Analysis of PHF-tau by ion spray mass spectrometry and peptide sequencing has revealed that 19 sites are at least partially phosphorylated (7, 13). Other posttranslational modifications of PHF-tau have been reported. For example, PHF-tau is both nonenzymatically glycated (14) and ubiquitinated (15-17). Recently, the presence of *N*-linked and (mucin-type) *O*-linked oligosaccharides on PHF-tau has also been suggested (18). However, the presence of these types of oligosaccharides on cytosolic proteins is highly controversial and has yet to be definitively documented for any protein (19, 20).

Cytoskeletal proteins such as neurofilaments (21), synapsins (22), and cytokeratins (23) are modified by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc). Recently, the high molecular weight MAPs MAP1, MAP2, and MAP4 have been shown to contain *O*-GlcNAc (24). This type of glycosylation is dynamic, consisting of a single monosaccharide, *N*-acetylglucosamine, glycosidically linked to side chain hydroxyls of serine (Ser) and threonine (Thr) residues on nuclear and cytoplasmic proteins of eukaryotes (25) (for reviews see Refs. 26-28). Although the exact function of this modification is not well understood, many lines of evidence suggest it to be an essential and abundant regulatory modification present in virtually all eukaryotes. For example, unlike other forms of protein glycosylation, *O*-GlcNAc turns over much more rapidly than the peptide backbone (29). *O*-GlcNAc is postulated to have modulatory functions similar to phosphorylation (30, 31). This hypothesis is supported by the finding that *O*-GlcNAc

bearing sites on certain proteins have been demonstrated to coincide with known sites of phosphorylation (32, 33). Furthermore, *O*-GlcNAcylation appears to regulate DNA binding by the p53 tumor suppressor protein (34) and to regulate protein synthesis by controlling the activity of EIF2-kinase (35, 36). Given phosphorylation's likely role in the formation of PHF-tau and the discovery that many cytoskeletal phosphoproteins are also *O*-GlcNAcylated (20, 37), we decided to investigate the possible glycosylation of normal tau. This study demonstrates that tau isolated from bovine brain is extensively modified by *O*-GlcNAc.

EXPERIMENTAL PROCEDURES

Materials--Uridine diphosphate (UDP)-[6-^{3}H]galactose (38 Ci/mmol) was obtained from Amersham Corp. [^{3}H]glucosamine (40 Ci/mmol) was from Du Pont NEN Bovine milk galactosyltransferase was from Sigma. Tosylphenylalanyl chloromethyl ketone-treated trypsin was from Worthington, and saccharide standards were from Dionex. Horseradish peroxidase-conjugated succinyl wheat germ agglutinin, *Triticum vulgaris* (sWGA-horseradish peroxidase), was from E-Y Laboratories. The tau monoclonal anti-body Tau2 (6) was a generous gift from Dr. L. Binder. All other reagents were of the highest commercial grade available.

Purification of Bovine Tau--Tau was purified to homogeneity from bovine brains (RJO Biologicals) as described previously (38, 39).

Galactosyltransferase Labeling of Tau Proteins and Separation of ^{3}H-Labeled Tau Glycopeptides--Tau polypeptides were probed for terminal GlcNAc using Gal-β(1,4)galactosyltransferase (galactosyltransferase) and UDP-[^{3}H]galactose as described by Roquemore *et al.* (40). Bovine tau was separated into individual isoforms by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Individual polypeptide bands were excised from the gel with a blade and extracted with 20 mM Tris, pH 7.5, 0.1% SDS. Radiolabeled tau glycopeptides were isolated from SDS-polyacrylamide gels after digesting with trypsin as described by Rosenfeld *et al.* (41). Glycopeptides were separated by reverse-phase high performance liquid chromatog-raphy (RP-HPLC) on a Rainin HPLC system equipped with a Vydac C18 column (4.6 X 250 mm) through a linear 2-h gradient of acetonitrile (0-40%) in buffer containing 0.1 M sodium perchlorate and 1% phosphoric acid, pH 2.1, at a flow rate of 1 ml/min (42). Absorbance of the eluent was monitored at 214 nm, and tritium was detected with an on-line Flo1β scintillation detector.

β-Elimination and Product Analysis--Purified bovine tau polypeptides were labeled with [^{3}H]galactose using galactosyltransferase followed by β-elimination and saccharide analysis which was performed as described previously (21, 43).

Stoichiometry Estimation of O-GlcNAc on Tau--Stoichiometry analysis was performed in triplicate as described by Roquemore *et al.* (44) with some modifications.

Briefly, purified bovine tau polypeptides in 20 mM MES, 1 mM EGTA, 1 mM $MgSO_4$ were desalted by gel filtration, precipitated with six volumes of acetone, and resuspended in deionized water, and protein levels determined by Micro bicinchoninic acid (45) or Amido Schwarz (46). A relative molecular mass of 46.2 kDa which represented an average of all bovine isoforms (3), was used in all calculations. Prior to hydrolysis, [^{3}H]-glucosamine (100,000 cpm) was added to each sample to determine percent recovery. Amino sugars were isolated on 0.5 ml of Dowex AG 50W-X8 ([H+] form) columns with 2 M HCl after washing with 20% methanol. The amount of glucosamine released from acid hydrolysis was quantified by peak areas of glucosamine standards on a Dionex CarboPac PA1 column. Identity of the glucosamine peak was confirmed by comigration with GlcNAc after re-*N*-acetylation (47).

SDS-Polyacrylamide Gel Electrophoresis and Autofluorography--Tau proteins were resolved on 8% SDS-polyacrylamide gels (48), stained with Coomassie Brilliant Blue R-250 (Bio-Rad), treated with 2-Hydroxybenzoic acid, dried, and exposed to X-OMAT film (Kodak).

WGA Lectin and Western Blot Analysis--Proteins were transferred to polyvinylidine difluoride (PVDF) or nitrocellulose membranes equilibrated in 25 mM Tris, pH 8.5, 190 mM glycine, and 20% methanol. After blocking the membranes with 5% bovine serum albumin in 125 mM NaCl, 20 mM Tris, pH 8.0, 0.1% Tween-20 (TBST), proteins were probed for GlcNAc with sWGA-horseradish peroxidase in TBST (1:5,000)

and developed with 3,3'-diaminobenzidine in the presence of hydrogen peroxide or by enhanced chemiluminescence. Blots were probed for tau with the monoclonal antibody Tau2 (1:10,000) followed by horseradish peroxidase-conjugated goat anti-mouse-IgG in TBST (1:2000) prior to developing.

Protein Determination--Protein concentrations were determined by Lowry *et. al.* (49), micro bicinchoninic acid assay (Pierce), or Amido Schwarz dye binding assay using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Galactosyltransferase Labels Terminal GlcNAc Moieties on Tau--The presence of terminal GlcNAc residues on bovine tau is demonstrated by labeling tau with [^{3}H]galactose using galactosyltransferase. Tau purified from bovine brain migrates on SDS-polyacrylamide gels as five polypeptides with relative molecular masses between 45 and 60 kDa (3, 38), and all are reactive with the tau monoclonal antibody Tau2 (Fig 1*A*). The identification of terminal GlcNAc residues with galactosyltransferase is highly specific (26), resulting in the formation of [^{3}H]Galβ(1-4)GlcNAc moieties on a glycoprotein. After incubating tau with galactosyltransferase in the presence of UDP-[^{3}H]galactose, labeling of all tau isoforms is observed (Fig. 1*B*). The radiolabel observed in *lane 2* is due to incorporation into bovine tau because there is no detectable labeling of the enzyme alone (*lane 1*).

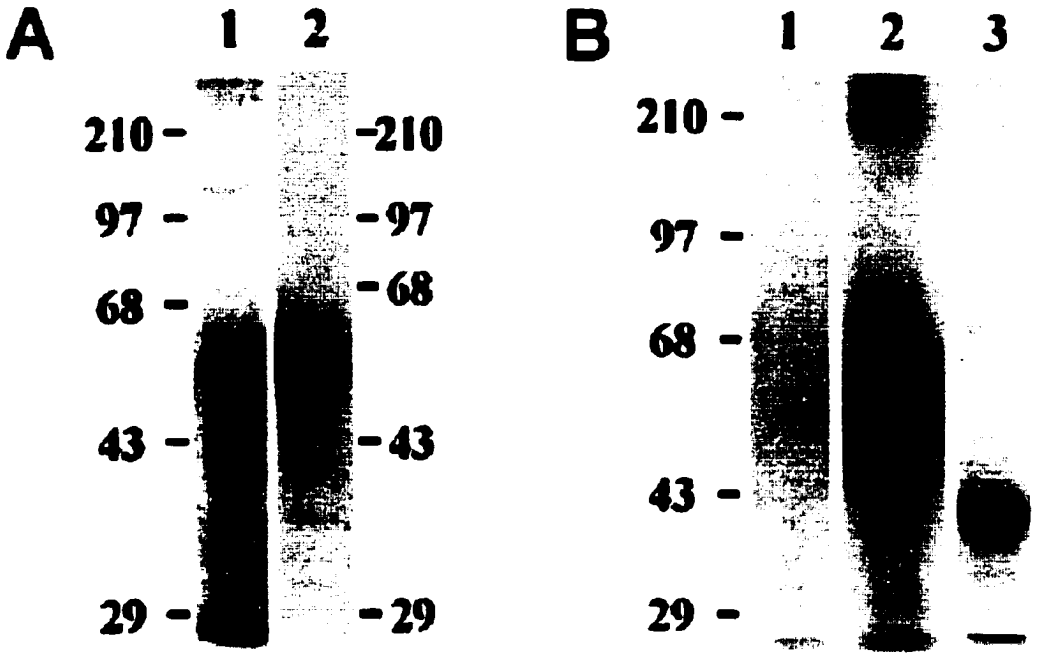


Fig. 1. **Galactosyltransferase labeling of purified bovine tau.** *A*. purified bovine tau polypeptides were resolved on 8% SDS-polyacrylamide gels and stained with Coomassie Blue (*lane 1*) or transferred to PVDF and probed with the monoclonal antibody Tau2 (*lane 2*). *B*, bovine tau polypeptides were labeled with [^{3}H]galactose as described under "Experimental Procedures." *Lane 1* contains galactosyltransferase only, and *lanes 2* and *3* are labeled bovine tau and ovalbumin, a positive control, respectively. Positions of molecular mass standards (kDa) are indicated in the margins.

The lectin sWGA also documents the presence of multiple terminal GlcNAc residues on bovine tau. sWGA binds specifically to GlcNAc (50). Lectin blots shown in Fig. 2 demonstrate that sWGA binds to purified bovine tau. The specificity of the sWGA lectin for GlcNAc is illustrated by effectively competing the lectin with 0.5 M GlcNAc, while 0.5 M galactose has little effect (Fig. 2). Together, these data indicate the existence of multiple terminal GlcNAc containing moieties on purified bovine tau.

Tau Is Glycosylated with O-linked GlcNAc--To determine the nature of the [^{3}H]-galactose-labeled residues on tau, labeled protein was first treated with peptide:*N*-glycosidase F(P*N*Gase F), an enzyme that specifically cleaves *N*-linked carbohydrates from the peptide backbone (51). Treatment of tau with P*N*Gase F did not remove the [^{3}H]galactose from tau, indicating that the carbohydrates on tau are not *N*-linked. In contrast, [^{3}H]galactosylated ovalbumin, a protein known to contain *N*-linked carbohydrates (52), is P*N*Gase F-sensitive (data not shown). The [^{3}H]galactose-labeled carbohydrates on tau are, however, susceptible to alkaline β-elimination (Fig. 3*A*), indicating that they are linked to the polypeptide via *O*-glycosidic linkages. TSK-Gel filtration of the [^{3}H]galactose-labeled compounds released by alkaline β-elimination demonstrate their migration as reduced disaccharides (Fig. 3*B*). Further analysis of the [^{3}H]galactose-labeled material by Dionex high pH anion exchange chromatography-pulsed amperometric detection shows that the labeled saccharides are derived from *O*-GlcNAc. Fig. 3*C* shows the co-migration of the [^{3}H]galactose-labeled compounds with Gal-β(1-4)GlcNAcitol, the

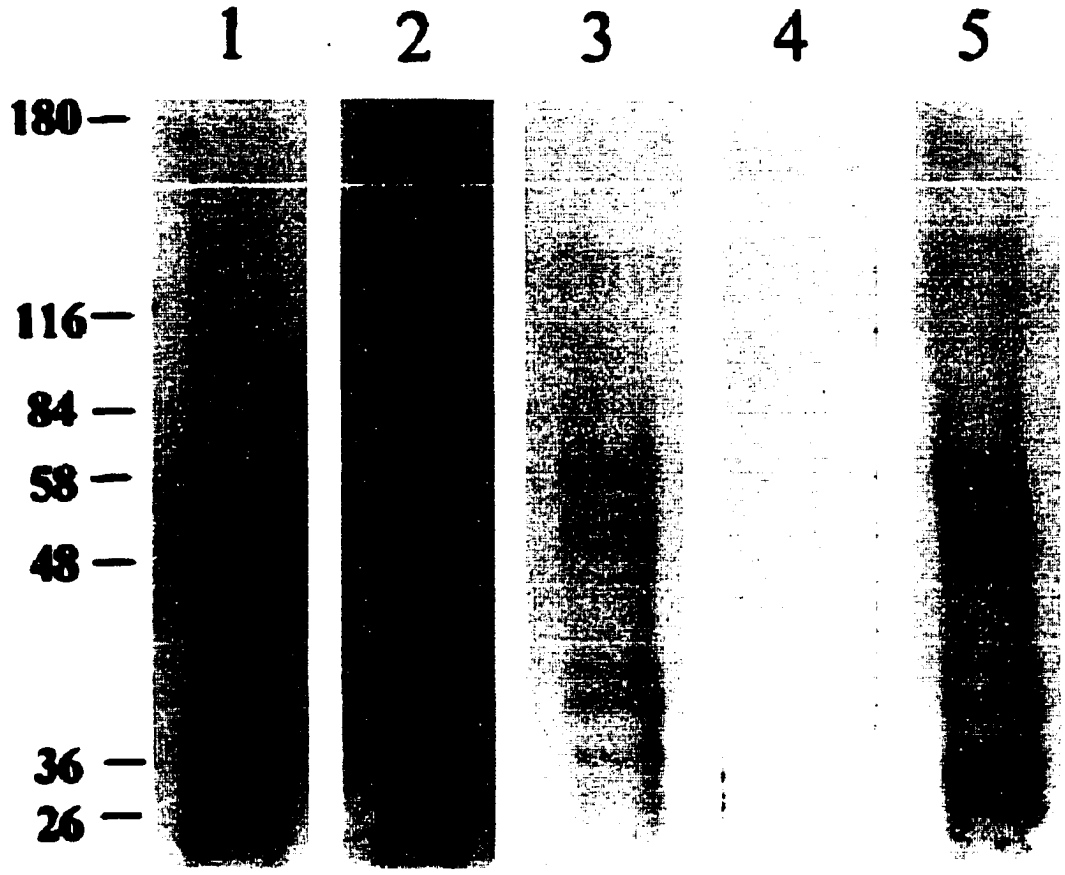
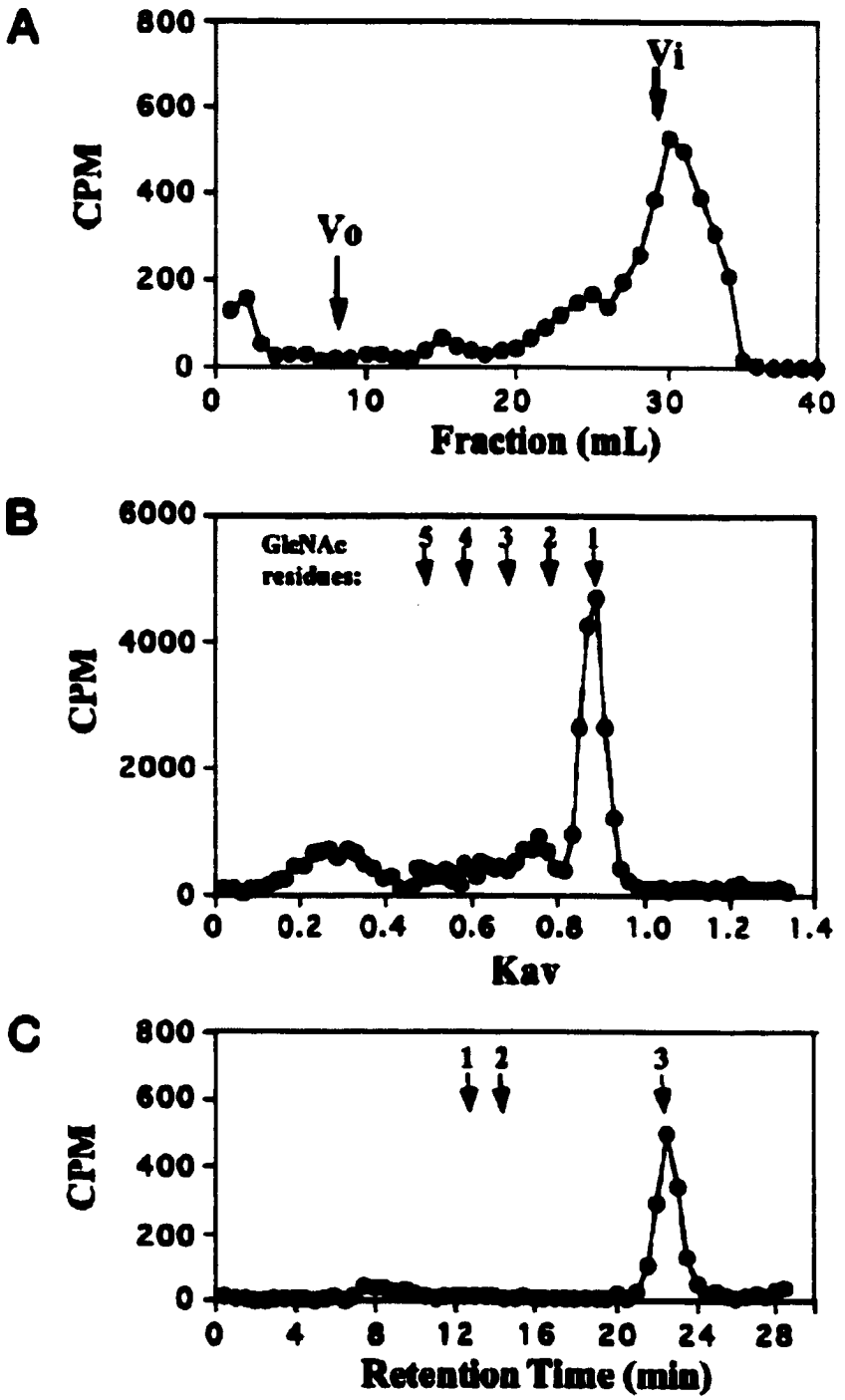


Fig. 2. **sWGA binding of *N*-acetylglucosamine on tau.** Equal amounts of purified bovine tau polypeptides were resolved on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with the monoclonal antibody Tau2 (*lane 1*) or with the lectin sWGA-horseradish peroxidase (*lanes 2-5*). Specificity of sWGA-horseradish peroxidase for GlcNAc is demonstrated by probing the membrane in the presence of 0.1 M GlcNAc (*lane 3*). *Lane 4* was incubated with 0.5 M GlcNAc, and *lane 5* was incubated with 0.5 M galactose. Positions of molecular mass standards (kDa) are indicated on the *left*.

Fig. 3. **Characterization of carbohydrate on tau proteins.** purified bovine tau was labeled with [^{3}H]galactose, and the β-elimination products were analyzed by: *A*, G-50 gel filtration chromatography. Vo, void volume; Vi, included volume. *B*, TSK-Fractigel chromatography. *Arrows* indicate the elution positions of [^{3}H]galactose-labeled GlcNAc polymers. Numbers correspond to GlcNAc residues. *C*, high pH anion exchange chromatography-pulsed amperometric detection analysis of β-elimination products on a Dionex CarboPAc-MA1 column. *Arrows 1* and *2* denote elution positions of Galβ1-3GalNAcitol and Galβ1-3GlcNAcitol standards, respectively. *Arrow 3* represents the elution time of Galβ1-4GlcNAcitol, the expected product of a galactose-labeled *O*-GlcNAc.

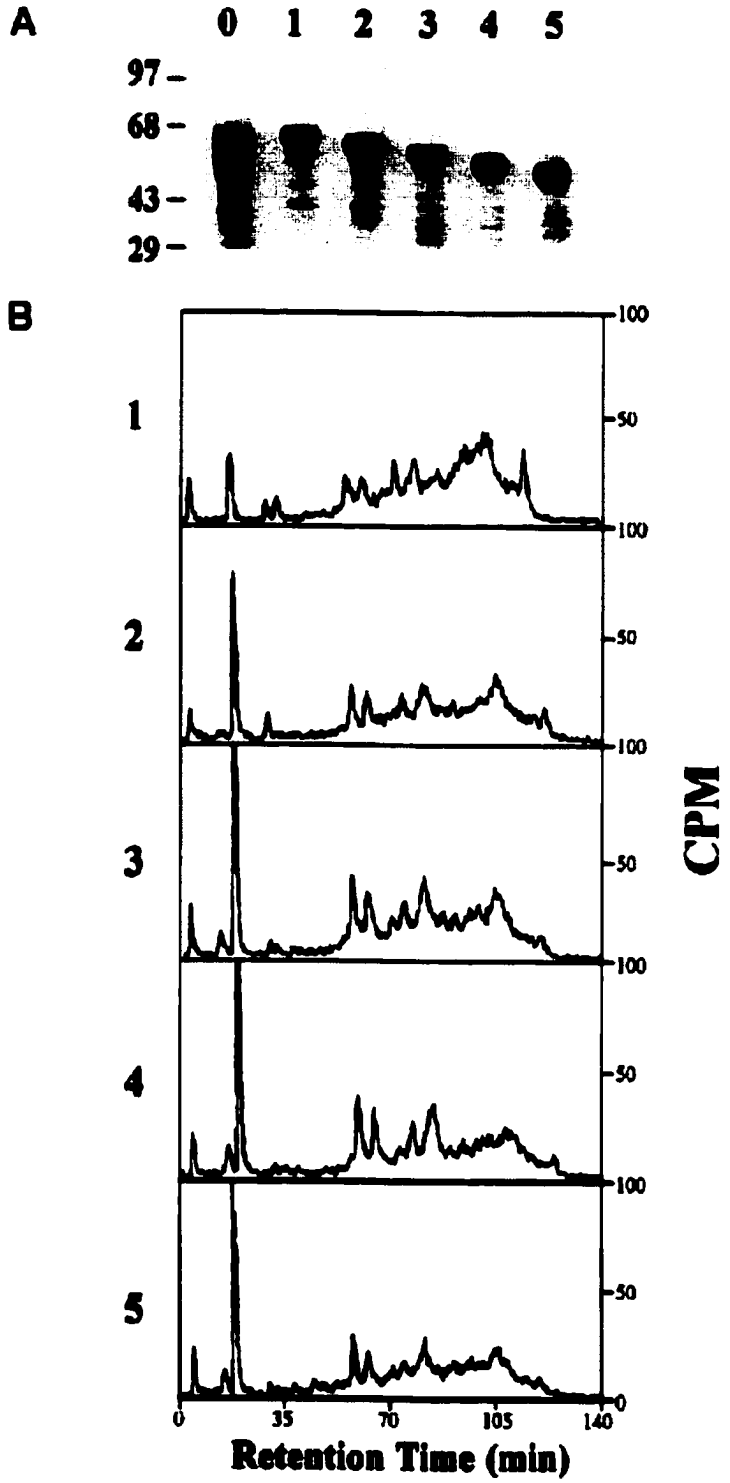


expected product of a galactose-labeled *O*-GlcNAc released by reductive, alkaline β-elimination. Thus, purified bovine tau is modified with *O*-GlcNAc.

All Bovine Tau Isoforms Are Extensively Glycosylated --To determine if any differences exist in the glycosylation of the different bovine tau isoforms, tryptic maps of each [^{3}H]galactose-labeled tau isoform were generated by RP-HPLC. The purified galactosyltransferase-treated polypeptide mixture was separated into its major species by SDS-polyacrylamide gel electrophoresis (Fig. 4*A*). In-gel trypsin digestion was performed on each isoform, and the peptides released from the gel were separated by RP-HPLC (Fig. 4*B*). The resulting profiles clearly suggest that the tau isoforms contain numerous sites of *O*-GlcNAcylation. The similar retention times for many of the peaks indicate that all of the isoforms appear to share similar sites of glycosylation. The minor differences between the different isoforms could represent some stoichiometry differences at individual sites of glycosylation or differential trypsin cleavage due to amino acid sequence diversity.

The number of glycosylation sites observed on tau appears to be relatively high as compared with certain other known *O*-GlcNAc-modified proteins (21, 29, 44). The stoichiometry of tau glycosylation was determined and found to be 4.2 +/- 0.9 mol *O*-GlcNAc/mol tau (n = 3 separate determinations). Initial site mapping data indicate that one major attachment site for *O*-GlcNAc on tau is localized to a domain involved in microtubule binding (2, 3). These data are further supported by the finding that a synthetic peptide based upon these mapping studies, and containing the tau sequence VKSKIGS-

Fig. 4. **Separation of [^{3}H]galactose-labeled bovine tau isoforms and RP-HPLC of trypsin digested polypeptides.** *A*, purified bovine tau (*lane 0*) and each of the purified individual bovine tau isoforms (*lanes 1-5*) were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed with the monoclonal antibody Tau2. Positions of molecular mass standards (kDa) are indicated at the *left*. *B*, RP-HPLC of tau [^{3}H]glycopeptides generated from trypsin digests. Numbers at the *left* of each profile correspond to respective proteins separated in panel *A*.



TENLKHQ is a substrate *in vitro* (data not shown) for the *O*-GlcNAc transferase (53). Further analysis of the *in vivo* tau glycosylation sites is being facilitated by the development of more sensitive methods (54, 55).

The high degree of glycosylation of bovine tau indicates a possible structural role for these glycoconjugates on tau. The *O*-GlcNAcylation of other MAPs, MAP1, MAP2, and MAP4, also suggests a role for *O*-GlcNAc in mediating the interactions of the MAPs with tubulin. Recently, tau has been shown to be associated with the neural plasma membrane (56) and also localized to the nucleus (57). The *O*-GlcNAcylation of tau may play a role in its subcellular localization. The demonstration of *O*-GlcNAc attachment to known phosphorylation sites of nuclear proteins, c-Myc (33) and RNA polymerase II (32), and the exclusive reciprocity of *O*-GlcNAc and phosphate on some *O*-GlcNAc modified proteins (33) suggest that tau glycosylation and phosphorylation may also be reciprocally related. Phosphorylation modulates tau's interactions with tubulin and the proteolysis of tau (58). Therefore, tau *O*-GlcNAcylation could potentially regulate tau degradation. The fact that *O*-GlcNAc has not been detected on PHFs (14), which are extensively phosphorylated at numerous sites (7), supports this hypothesis concerning the reciprocal nature of these two modifications. Thus, these data suggest the intriguing possibility that in Alzheimer's disease (AD), PHF-tau formation may result from the defective *O*-GlcNAcylation of tau, thus allowing or even promoting abnormal tau hyperphosphorylation. *O*-GlcNAc's putative involvement in the etiology of AD is made more intriguing by the recent finding of *O*-GlcNAc on the cytoplasmic domain of the β-amyloid precursor protein (59) and by the direct observation of altered *O*-GlcNAcylation in

the brains of AD patients (60). Future investigations will directly evaluate the role(s) of *O*-GlcNAcylation in microtubule assembly and organization. One obvious implication predicted from the present study is that inhibitors of enzymes specific for the removal of *O*-GlcNAc (61) could prevent PHF-tau formation by not allowing tau hyperphosphorylation to occur.

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DISCUSSION OF ADDITIONAL SIGNIFICANT FINDINGS

Glycosylation Site Analysis of Tau

Summary--Tau is a family of closely related microtubule-associated proteins (MAPs) important for regulating microtubule stability in neurons. Additionally, abnormally hyperphosphorylated tau is the primary component of neurofibrillary tangles (NFTs) associated with neurodegenerative diseases such as Alzheimer's disease (AD). Previously, we described the extensive glycosylation of bovine tau by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), a dynamic modification that is sometimes reciprocal to phosphorylation. For the identification of *O*-GlcNAcylation sites on tau, bovine tau polypeptides were digested with trypsin, [^{3}H]galactosylated by Galβ(1,4)galactosyltransferase, and glycopeptides were isolated by several rounds of reverse-phase high pressure liquid chromatography (RP-HPLC). Two major radioactive peaks were further purified and subjected to gas-phase sequencing, yielding the peptide sequences IGSTENLK and SKIGSTENLK, an incompletely digested tryptic peptide. These sequences lie within the first microtubule-binding repeat of tau and contain the amino acid Ser-262, a paired helical filament (PHF)-tau phosphorylation site in AD brain. Glycosylation of this region was verified by *in vitro O*-GlcNAcylation of the peptide CVKSKIGSTENLKHQ by rat brain extract. An additional peptide, SPVVSGDTSPR, located within the carboxyl-terminus of tau was also a substrate for *in vitro O*-GlcNAcylation. Manual Edman degradation of this C-terminal peptide identified the site of glycosylation as

Ser-400, a glycogen synthase kinase 3β (GSK3β) site of phosphorylation and PHF-tau phosphorylation site unique to Alzheimer's brain. The reciprocal nature of the glycosylation and phosphorylation at these biologically significant amino acids are discussed.

Experimental Procedures--Materials--Uridine diphosphate (UDP)-[6-^{3}H]galactose (38 Ci/mmol) was obtained from Amersham Corp. UDP-[6-^{3}H]-*N*-acetylglucosamine (18.9 Ci/mmol) was from DuPont-NEN. Frozen bovine and rat brains were from RJO Biologicals. Bovine milk galactosyltransferase was from Sigma and autogalactosylated as previously described (70). Tosylphenylalanyl chloromethyl ketone-treated trypsin was from Worthington. Sep-Pak C18 cartridges were from Millipore. The standard peptide YSDSPSTST and two tau-derived peptides, CVKSKIGSTENLKHQ and SPPVSGDTSPR, were synthesized on a Rainin automated peptide synthesizer and purified by RP-HPLC. All other reagents were of the highest commercial grade available.

Trypsin Digestion of Purified Bovine Tau and Galactosyltransferase Labeling of Tryptic Peptides--Bovine tau polypeptides were resolved on a 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel, stained with aqueous 0.1% Coomassie Brilliant Blue R-250, excised with a blade, and extracted with five changes of 20 mM Tris, pH 7.5, and 0.1% SDS. Proteins were lyophilized, resuspended in 8 M urea, 0.4 M NH_4HCO_3, pH 8.0, and reduced and alkylated by the addition of 0.1 M DTT and 0.1 M iodoacetamide. Samples were subsequently desalted by G50-sephadex gel filtration chromatography, precipitated with ethanol, and digested with trypsin (1:50) overnight at 37°C in 50 mM

NH_4HCO_3, pH 8.0. Peptides were galactosyltransferase labeled as previously described (71), isolated on Sep-Pak C18 cartridges, and analyzed by RP-HPLC.

RP-HPLC--Tritium-labeled glycopeptides were isolated by several rounds of RP-HPLC on a Rainin model (48-00XL) HPLC system equipped with either a Vydac 5-μm C18 column (4.6 X 250 mm) or a Vydac narrow-bore C18 column (2.1 X 150 mm). Glycopeptides were bound to the column in either 0.1% trifluoroacetic acid or 0.1% phos-phoric acid containing 0.2 M sodium perchlorate (72) and eluted with an increasing gradient of acetonitrile. Elution of peptides was monitored by absorbance at 214 nm, and glycopeptides were detected by liquid scintillation spectrometry of an aliquot of each fraction.

Gas-phase Sequencing and Manual Edman Degradation--The primary sequences of HPLC-purified glycopeptides were determined by gas-phase automated Edman degradation on a Porton Instruments (Tarzana, CA) model PI 2090E microsequencing system equipped with an on-line PTH amino acid analyzer. Manual Edman degradation (73) of tau peptides glycosylated *in vitro* was performed as previously described (74).

O-GlcNAcylation of Tau Synthetic Peptides--The *O*-GlcNAcylation of peptides was performed as previously described (75) with minor modifications. Briefly, frozen rat brains were homogenized in a Dounce homogenizer in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, and centrifuged at 27,500 x g for 35 min at 4°C. Proteins were precipitated from brain homogenates by the addition of 75% ammonium sulfate followed by incubation for 30 min at 4°C and centrifugation at 12,000 x

g for 30 min. Pellets were resuspended in 20% glycerol, 10 mM Tris, pH 8.0, 1 mM DTT prior to desalting and assaying.

Results and Discussion--Purified bovine tau polypeptides were first trypsinized to gain accessibility to any *O*-GlcNAc residues on the proteins for subsequent galactosyltransferase labeling. The *O*-GlcNAcylated glycopeptides labeled with [^{3}H]galactose by galactosyltransferase were resolved by RP-HPLC on a C18 column at pH 2.1 in the first dimension. As illustrated in Fig. 1, two major radioactive peaks, designated *peak a* and *peak b*, as well as numerous other unresolved radiolabeled peaks, were detected. The radioactive fractions containing *peak a* and *peak b* were subsequently resolved in a second dimension on a C18 column at pH 7.0, and in a third dimension at pH 2.1.

Aliquots of [^{3}H]galactose-labeled glycopeptide from the third dimension of the purifications of *peak a* and *peak b* were subjected to gas-phase sequencing. The relative abundance of amino acids in each cycle suggested that both *peak a* and *peak b* were essentially composed of the peptide sequences IGSTENLK and SKIGSTENLK, respectively (Fig. 1). The second sequence derived from *peak b*, SKIGSTENLK, was a tau tryptic peptide containing amino acids 258-267, according to the numbering of the longest isoform of human tau (27). *Peak b* also contains the sequence found in *peak a*, IGS-TENLK, containing tau amino acids 260-267, suggesting *peak b* arose due to the in-complete trypsin digestion of tau before Lys-259.

For the identification of the site of *O*-GlcNAc attachment, aliquots of each [^{3}H]-galactosylated-glycopeptide were coupled to an aryl-amine disk and subjected to manual Edman degradation. After washing the membranes with methanol, trifluoroacetic acid, and dH$_2$O, an insufficient amount of radioactivity was bound, prohibiting further ident-

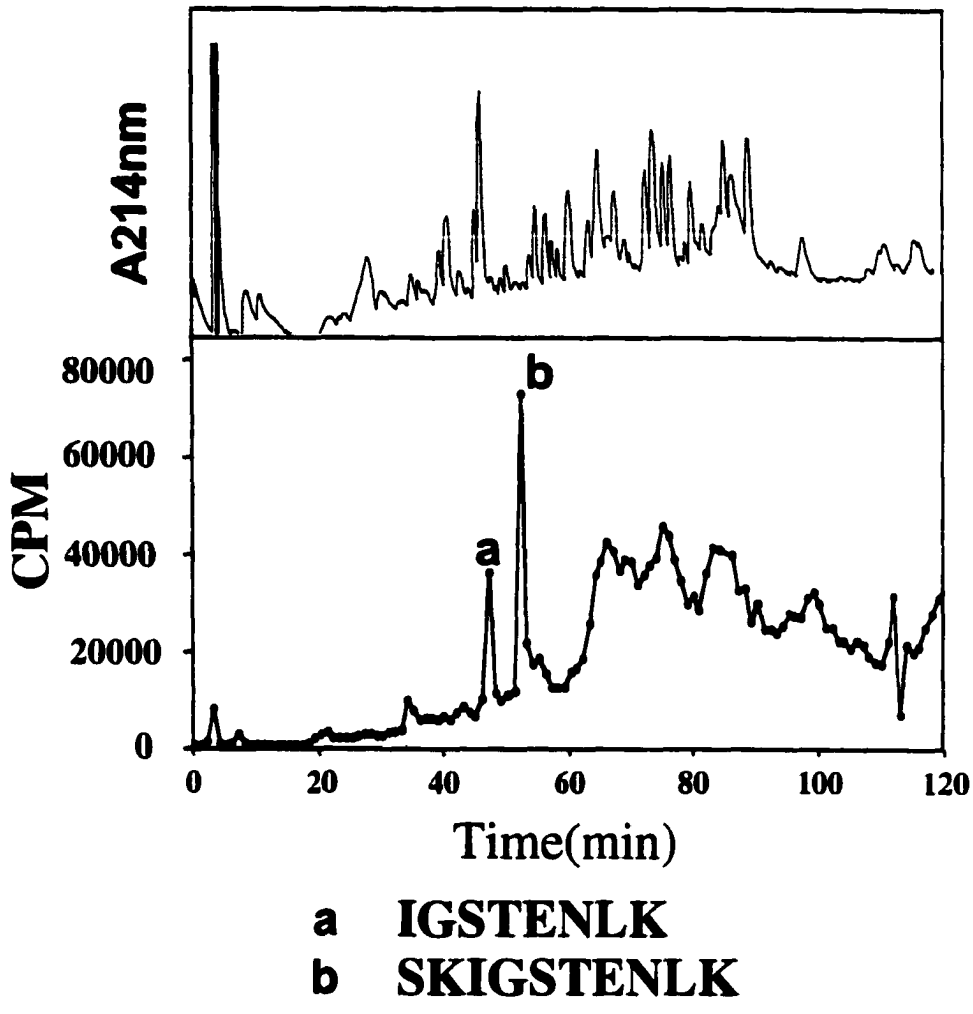


Fig.1. **Tau is glycosylated within the first microtubule-binding repeat.** RP-HPLC analysis of bovine tau tryptic glycopeptides radiolabeled by galactosyltransferase and UDP-[^{3}H]galactose. Peptides were resolved on a C18 column through a linear 2-h gradient of acetonitrile (0-40%) in 0.2 M sodium perchlorate, 1% phosphoric acid, pH 2.1, at a flow rate of 1 ml/min. Two major radioactive peaks (*a*, *b*) were further purified and subjected to gas-phase sequencing, yielding the peptide sequences IGSTENLK (*a*) and SKIGSTENLK (*b*).

ification of the sites of glycosylation. Nevertheless, in order to verify the *O*-GlcNAcylation of this region, a peptide containing the tau amino acids 255-269 and both sequences identified from *peak a* and *peak b*, CVKSKIGSTENLKHQ, was synthesized. The synthetic tau peptide (3 mM) was incubated with rat brain extract in the presence of [^{3}H]-UDP-GlcNAc in an *in vitro O*-GlcNAc transferase assay. Within 30 min, the tau synthetic peptide incorporated more than 5,000 cpm of [^{3}H]GlcNAc over enzyme background (Fig. 2), indicating this region within tau is a substrate for the rat brain *O*-GlcNAc transferase and therefore is modified by *O*-GlcNAc.

Due to the difficulties associated with the identification of *in vivo* sites of *O*-GlcNAcylation such as repetitive losses associated with traditional glycopeptide purification schemes and the success of the *in vitro O*-GlcNAcylation of the tau synthetic peptide, another tau peptide was synthesized and tested for its ability to be glycosylated in this assay. To assess the *O*-GlcNAcylation of a region within the C-terminus of tau, the peptide SPVVSGDTSPR, corresponding to amino acids 396-406 according to the longest isoform of human tau, was incubated with rat brain extract and UDP-[^{3}H]-GlcNAc. The peptide SPVVSGDTSPR was *O*-GlcNAcylated *in vitro* and was subsequently coupled to an aryl-amine disk and subjected to manual Edman degradation for identification of the site of attachment (Fig. 3). An overwhelming majority of the [^{3}H] counts were released after the fifth cycle of cleavage, indicating that Ser-400 was glycosylated with *O*-GlcNAc in this *in vitro* assay.

Serine-400 (Ser-400) is phosphorylated in PHF-tau (44) and is likewise a substrate for phosphorylation *in vitro* by GSK3β /(Tau protein kinase I) after heparin potentiation (76). Additionally, GSK3β phosphorylates tau at residues Ser-46, threonine-50 (Thr-50), Ser-199, Ser-202, Thr-205, Thr-231, Ser-396, Ser-404, and Ser-413. The

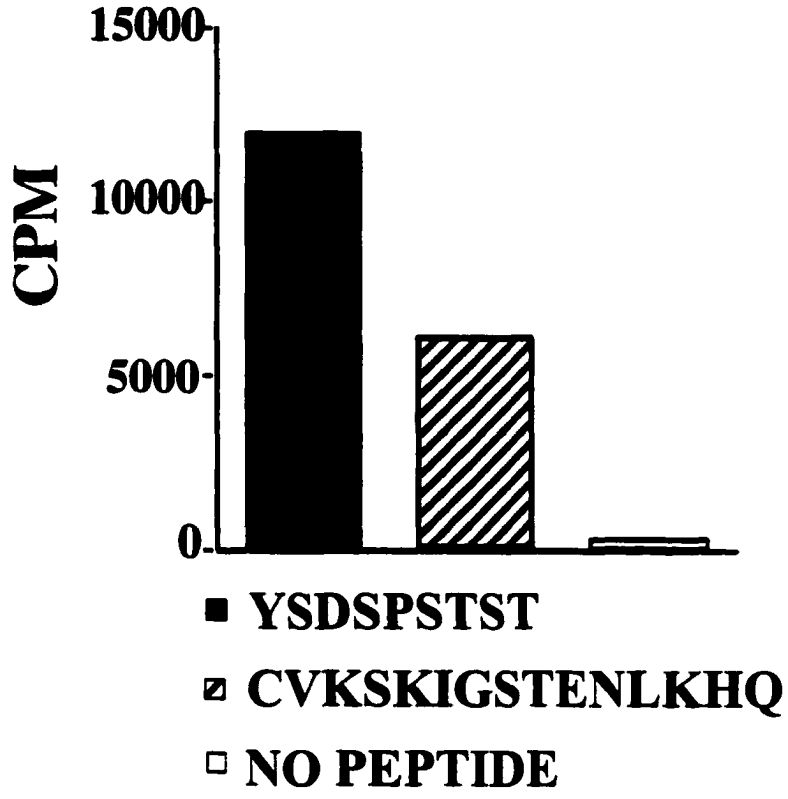


Fig. 2. ***In vitro* O-GlcNAcylation of tau synthetic peptide within the first microtubule-binding repeat.** Synthetic peptides (3 mM) were incubated in the presence of rat brain extract and UDP-[^{3}H]-GlcNAc for 30 min at room temperature. Peptides were separated from unincorporated label by isolation on SP-sephadex in 50 mM ammonium formate and elution with 0.5 M NaCl. Data are representative of duplicate experiments.

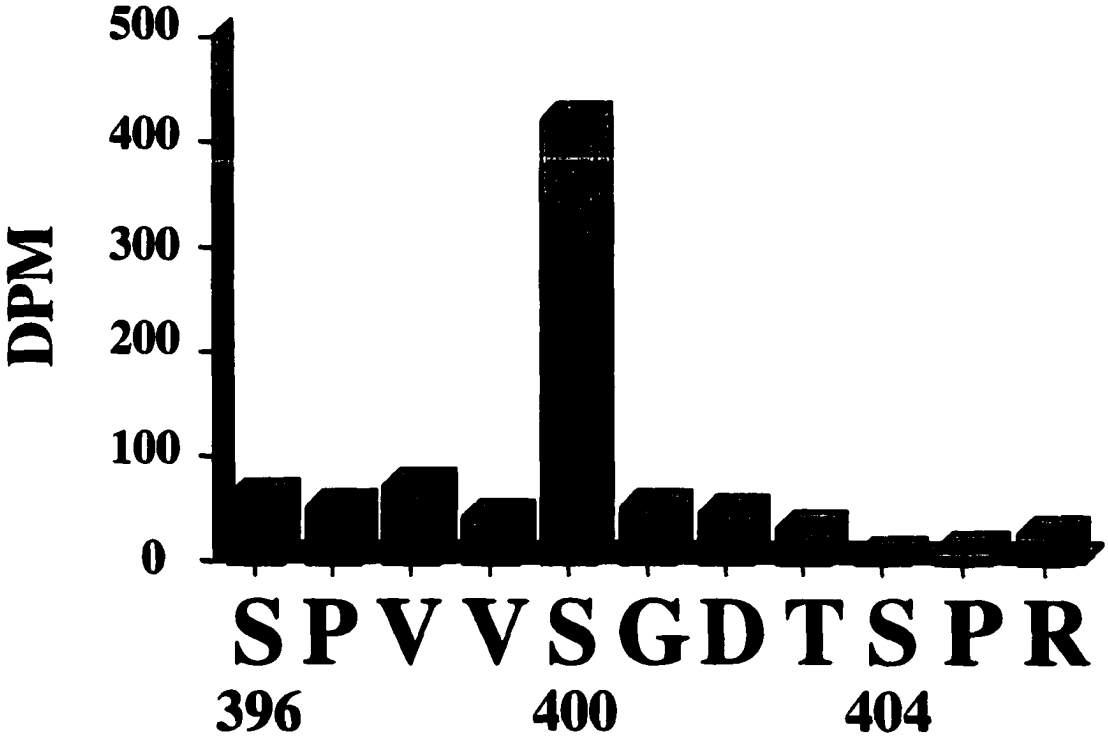


Fig. 3. **Manual Edman degradation of C-terminal tau glycopeptide.** A synthetic peptide corresponding to amino acids 396-406 from tau (according to the long-est human isoform) was *O*-GlcNAcylated *in vitro* by the addition of rat brain extract and UDP-[^{3}H]-GlcNAc. The [^{3}H]glycopeptide was coupled to an aryl-amine disk and subjected to subsequent rounds of Edman degradation.

importance of the phosphoryation of tau by GSK3β and the putative involvement of GSK3β in the etiology of AD has been demonstrated by several groups. For instance, GSK3β co-purifies with the PHFs isolated from the NFTs of AD brain (77, 78) and associates with familial AD-associated presenilin-1 (PS1) mutants (79). Furthermore, transfection of 3T3 and COS-7 fibroblasts (80, 81) and CHO cells with GSK3β and tau promotes the GSK3β-mediated phosphorylation of tau, reduces tau-mediated microtubule bundling, and diminishes tau's affinity for microtubules (82). Thus, the same-site occupancy of *O*-GlcNAc and phosphate at Ser-400 may have profound effects on the function of tau. For example, the *O*-GlcNAcylation of tau at Ser-400 could regulate the GSK3β-mediated phosphorylation of tau and, as such, influence the association of tau with microtubules controlled by GSK3β.

The tau sequence, SKIGSTENLK, identified from the gas-phase sequencing of [^{3}H]galactosylated glycopeptide *peaks a* and *b*, lies within the first microtubule-binding repeat and contains a known *in vivo* phosphorylation site, Ser-262 (83). As mentioned, this region was also *O*-GlcNAcylated by rat brain extract *in vitro*. Tau is phosphorylated on Ser-262 by a microtubule-affinity regulating kinase (MARK) (41), phosphorylase kinase (84), and protein kinase A (PKA) (85) *in vitro*, and phosphorylation of tau at Ser-262 is elevated in AD. When tau is phosphorylated at Ser-262, tau's affinity for microtubules is drastically reduced (32). Investigators have also demonstrated that the phosphorylation of tau at Ser-262 is also required for cell process formation (86). Due to the fact the microtubule-binding region containing Ser-262 is *O*-GlcNAcylated, Ser-262 could be reciprocally modified with both *O*-GlcNAc and phosphate. Another possibility may include the *O*-GlcNAcylation of the adjacent site, Thr-263. Regardless, *O*-

GlcNAcylation of this region could block the phosphorylation of tau at Ser-262 by steric hindrance or direct reciprocity and direct the association of tau with microtubules. Ultimately, the *O*-GlcNAcylation of tau within the first microtubule-binding repeat and at Ser-400 suggests that *O*-GlcNAc may be an important regulator of tau functions in microtubule binding and stability, and defects in the *O*-GlcNAcylation of tau may contribute to the abnormal hyperphosphorylation of tau found in PHFs.

Phosphorylation of Tau in Reticulocyte Lysates

Summary--The *in vitro* transcription and translation of proteins in rabbit reticulocyte lysates is a rapid and efficient method for the analysis of proteins (87, 88) and their post-translational modifications such as *O*-GlcNAcylation (89, 90) and phosphorylation (91, 92). The gene for the longest human isoform of the MAP-tau (htau-40) (27) was sub-cloned into the expression vectors pcDNA 3.1 and pCITE for characterization in this cell-free system. After *in vitro* transcription and translation of tau in the presence of [^{35}S]methionine, lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography for confirmation of the expected translated product's corresponding molecular mass. Incubation of reticulocyte lysates containing *in vitro* translated tau at 37°C caused an upward shift in mobility of the radiolabeled proteins on SDS-polyacrylamide gels, indicating possible abnormal post-translational processing. Although β-D-*N*-acetylhexosaminidase treatment of lysates expressing *in vitro* translated tau had no effect on the electrophoretic mobility of tau, the gel retardation of tau could be reversed by incubation with either alkaline phosphatase or recombinant protein phosphatase, suggesting the intrinsic phosphorylation of tau. Further analysis of *in vitro* transcribed and translated tau by two-dimensional non-equilibrium pH gradient gel-

electrophoresis (2D-NEPHGE) demonstrated a phosphatase-dependent shift in isoelectric point confirming the endogenous phosphorylation of tau in reticulocyte lysates. The rapid convenience of this system may provide a useful means of identifying likely protein kinases responsible for the *in vivo* phosphorylation of normal and abnormally hyperphosphorylated tau.

Experimental Procedures--Materials--Plasmids pBRK-174 and pcDNA 3.1 containing the longest human tau isoform, T4L, were generous gifts from Dr. Gail V. W. Johnson. The expression vectors pcDNA 3.1 and pCITE were obtained from Invitrogen and Novagen, respectively. Human tau clone htau40 (T4L) was a generous gift of Michel Goedert. *In vitro* transcription/translation TNT rabbit reticulocyte expression systems were from Promega. Alkaline phosphatase (Type III-L) was from Sigma. [^{35}S]-Methionine (>1000 Ci/mmol) was from Amersham Pharmacia. β-D-*N*-acetylhexosaminidase was from V-Labs (Covington, LA). Recombinant lambda protein phosphatase was from New England Biolabs. Protein kinase inhibitors were from Calbiochem. All other reagents were of the highest commercial grade available.

Purification of recombinant Tau from *E. coli*--*E. coli* strain BL21DE3 was transformed with the inducible expression vector pBRK-174 containing the longest isoform of human tau (T4L), and tau was purified as previously described (93), with the additional modifications. Bacterial cultures were grown at 37°C until the optical density at 600 nm was (0.4-0.6), induced with 0.5 mM IPTG for 2 h, and isolated by centrifugation at 5,000 x g for 10 min at 4°C. Bacterial pellets were resuspended in 50 mM Tris, pH 7.5, PICS 1, PICS 2, 1 mM PMSF, 1 mM EGTA, 1 mM EDTA, and 5 mM DTT, and

centrifuged for 10 min at 17,000 x g at 4°C. The supernatant was isolated and brought to 0.75 M NaCl and 5 mM DTT, boiled for 10 min, incubated on ice for 10 min, and centrifuged for 30 min at 17, 000 x g at 4°C. The heat-stable extract was brought to 45% ammonium sulfate, incubated for 18 h at 4°C, centrifuged at 20,000 x g for 20 min, and resuspended in and dialyzed against 20 mM MES, pH 7.2, 1 mM EGTA, and 1 mM $MgSO_4$.

In vitro Transcription and Translation--Plasmids pcDNA 3.1 containing T4L and pGEM containing the cDNA for nucleoporin p62 were isolated from *E. coli* strain XL1-Blue using the alkaline lysis maxi-prep system from Qiagen as described (94). [^{35}S]Methionine-labeled proteins were generated by *in vitro* transcription and translation according to manufacturer's instructions.

SDS-Polyacrylamide Gel Electrophoresis and 2D-NEPHGE--For electrophoresis on 10% SDS-polyacrylamide gels, 5 µl of reticulocyte lysate were boiled in Laemmli buffer (95) and electrophoresed at 100 V for 2.5 h. For NEPHGE, samples were lyophilized and resuspended in 9.5 M urea, 2% NP-40, 5% 2-mercaptoethanol, 1.5% ampholytes (pH 5-8), and 0.5% ampholytes (pH 3.5-10) and run at 500 V reversed polarity for 95 min in the first dimension as described (96). Tube gels were overlaid with SDS-polyacrylamide gel electrophoresis running buffer and electrophoresed in the second dimension. Radioactive spots were visualized by autoradiography and aligned by internal 2D-isoelectric focusing standards.

Phosphatase Digestion--Reticulocyte lysates were digested with 0.1 U of alkaline phosphatase,Type III-L (Sigma), for 18 h at 37°C in 50 mM Tris, pH 8.3, 2 mM $MgCl_2$, 0.1 mM $ZnCl_2$, PICS 1, and PICS 2. Lysates digested with lambda recombinant protein phosphatase were treated following manufacturer's instructions.

β-D-*N*-acetylhexosaminidase Digestion--After a 2-h *in vitro* transcription/translation reaction, lysates were digested with 0.3 U of jack-bean β-D-*N*-acetylhexosaminidase for 18 h at 37°C in 50 mM citrate, pH 5.5, PICS 1, PICS 2, and 1% aprotinin.

Results and Discussion--In order to evaluate the glycosylation of tau in other systems, the *in vitro* transcription and translation reaction was chosen because of its rapid ability to produce a radiolabeled protein in a mammalian cell lysate. After expression of the longest human isoform of tau (T4L) from the pcDNA 3.1 vector in the reticulocyte lysate, two major radioactive polypeptides of equal intensity with an approximate molecular mass of 65 kDa were identified (Fig. 4). T4L translated from the p-CITE vector yielded predominately one radioactive band (data not shown) suggesting the extra band was likely generated due to an upstream start site that is vector dependent. However, when *in vitro* translated tau was incubated for 18 h at 37°C, multiple higher molecular weight bands exhibiting a shift in gel mobility were observed (Fig. 5). Hypothesizing that the retarded mobility of *in vitro* translated tau was due to increased protein *O*-GlcNAcylation; we digested lysates with β-D-*N*-acetylglucosaminidase followed by SDS-polyacrylamide gel electrophoresis. The nucleoporin p62, a highly *O*-GlcNAcylated protein, and para-nitrophenyl-*N*-acetylglucosamine (pNP-GlcNAc), were digested in parallel as positive controls. Digestion with β-D-*N*-acetylhexosaminidase

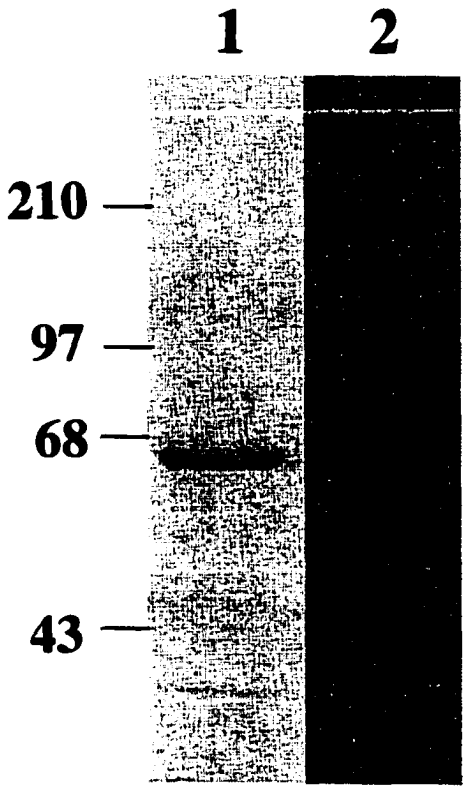
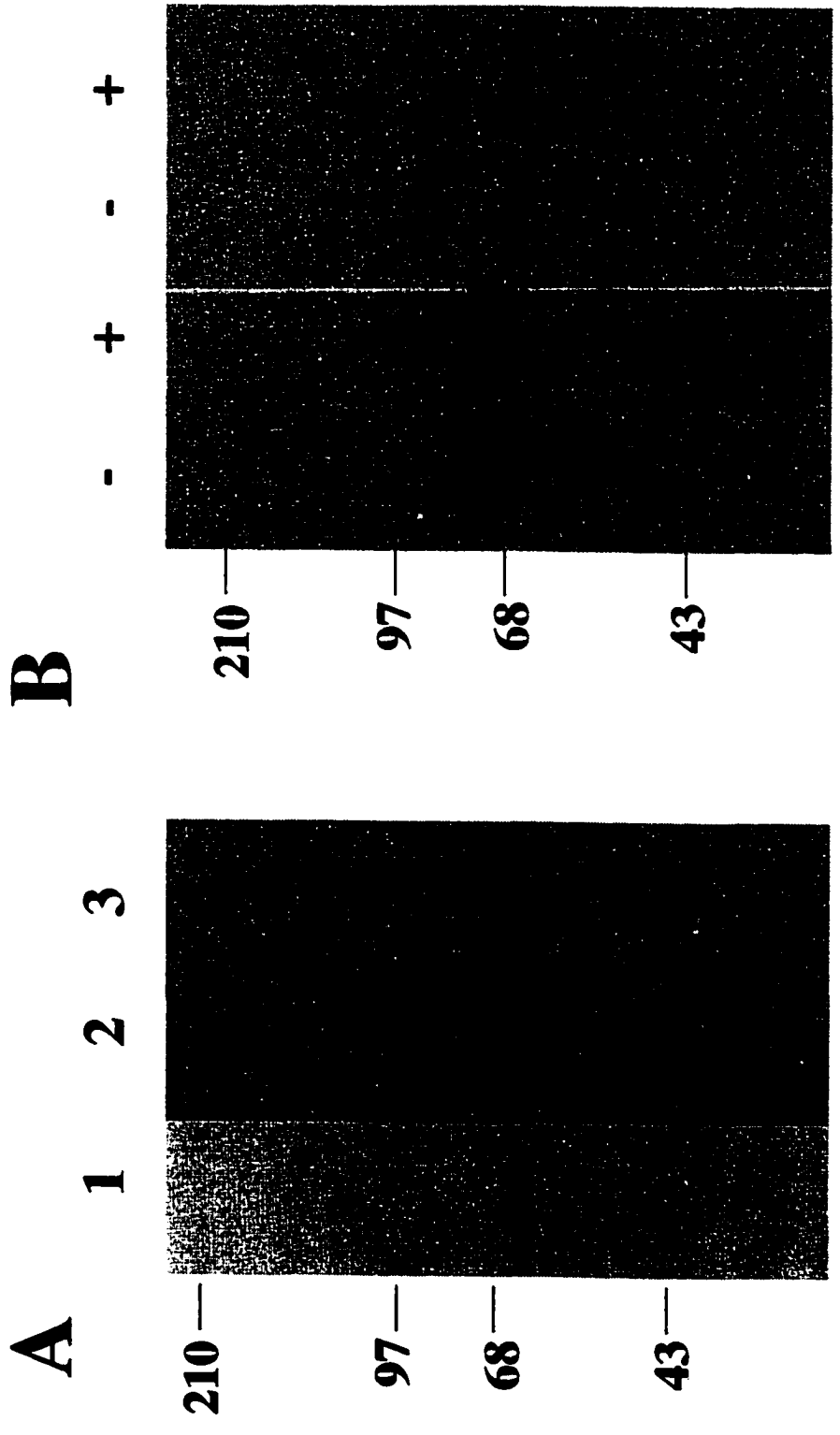


Fig.4. **Expression of tau in *E. coli* and rabbit reticulocyte lysates.** The longest human tau isoform (T4L) was subcloned into the vector pcDNA3.1, expressed and purified from *E. coli*, resolved on a 10% SDS-polyacrylamide gel, and stained with Coomassie Blue (*lane 1*). T4L was also transcribed and translated in the presence of [^{35}S]-methionine in rabbit reticulocyte lysate, resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography (*lane 2*). Molecular mass standards (kDa) are indicated at *left*.

Fig. 5. **β-D-*N*-acetylhexosaminidase digestion of *in vitro* transcribed and translated tau.** *A*, T4L purified from *E. coli* (*lane 1*) and *in vitro* translated tau (*lane 2*) and nucleoporin p62 (*lane 3*) were electrophoresed on 10% SDS-polyacrylamide gels. *B*, *in vitro* translated tau and p62 were incubated for 18 h at 37°C in the absence (-) or presence of (+) β-D-*N*-acetylhexosaminidase, electrophoresed on 10% SDS-polyacrylamide gels, and visualized by autoradiography. Molecular mass standards (kDa) indicated in margins.



had no effect on the mobility of tau but did shift p62, indicating that the shift of tau to higher molecular species may not be due to glycosylation (Fig. 5).

Because PHF-tau isolated from the NFTs in Alzheimer's brain is abnormally hyperphosphorylated and exhibits retarded mobility on SDS-polyacrylamide gels (97, 98), the phosphorylation of tau in reticulocyte lysates was investigated. Incubation of *in vitro* translated tau with alkaline phosphatase for 18 h at 37°C restored tau to its initial molecular mass on SDS-polyacrylamide gels (Fig. 6*A*) following *in vitro* transcription and translation. The increased mobility of tau was not due to proteolysis because recombinant protein phosphatase digestion had a similar effect (Fig. 6*B*), suggesting tau is likely phosphorylated in reticulocyte lysates.

For verification of the phosphorylation of tau in reticulocyte lysates, *in vitro* translated tau was analyzed by 2D-NEPHGE. Both abnormally hyperphosphorylated tau purified from Alzheimer's brains (99, 100) and recombinant tau phosphorylated *in vitro* by Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) or PKA (101) have acidic isoelectric points as analyzed by their migration on 2D-NEPHGE. Radiolabeled tau expressed in reticulocyte lysates exhibits retarded mobility on SDS-polyacrylamide gels and a shift in isoelectric point (pI) on 2D-NEPHGE after incubation for 18 h at 37°C. The shift in pI of the tau is due to phosphorylation because digestion with alkaline phosphatase shifts the radiolabeled tau species toward its native pI (Fig. 7). Thus, tau is phosphorylated by a protein kinase(s) endogenous to reticulocyte lysate that alters its pI and impairs its mobility on SDS-polyacrylamide gels.

The phosphorylation of tau by numerous protein kinases is well documented (23). Tau is phosphorylated by (Ser/Thr)-proline-directed protein kinases such as GSK-3β (86), CaMKII (101), cdc2 kinase (102), and MAP kinase (103). Phosphorylation of

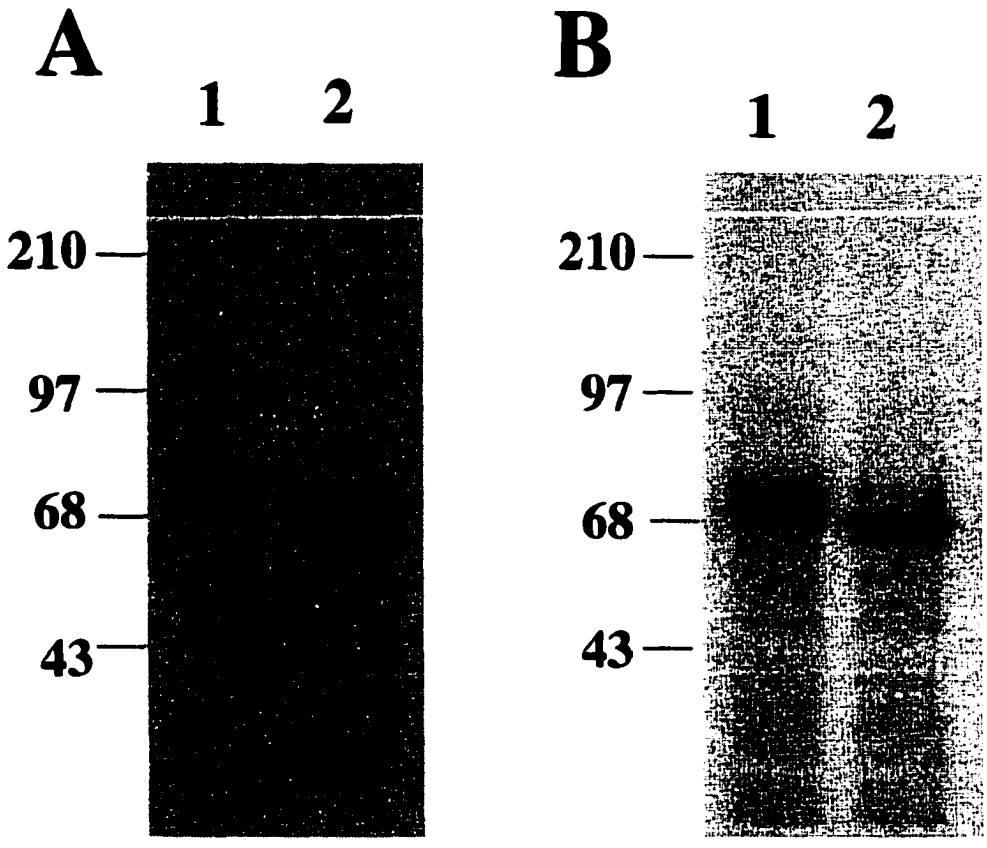


Fig. 6. **Phosphatase digestion of tau expressed in reticulocyte lysates.** *A*, T4L expressed in pcDNA3.1 was transcribed and translated in reticulocyte lysate in the presence of [^{35}S]methionine, incubated for 18 h at 37°C in the absence (*lane 1*) or presence (*lane 2*) of alkaline phosphatase, followed by electrophoresis on 10% SDS-polyacrylamide gels. *B*, T4L expressed in the pCITE vector was likewise incubated for 18 h at 37°C in the absence (*lane 1*) or presence (*lane 2*) of recombinant protein phosphatase, resolved on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. Molecular mass standards (kDa) indicated at *left*.

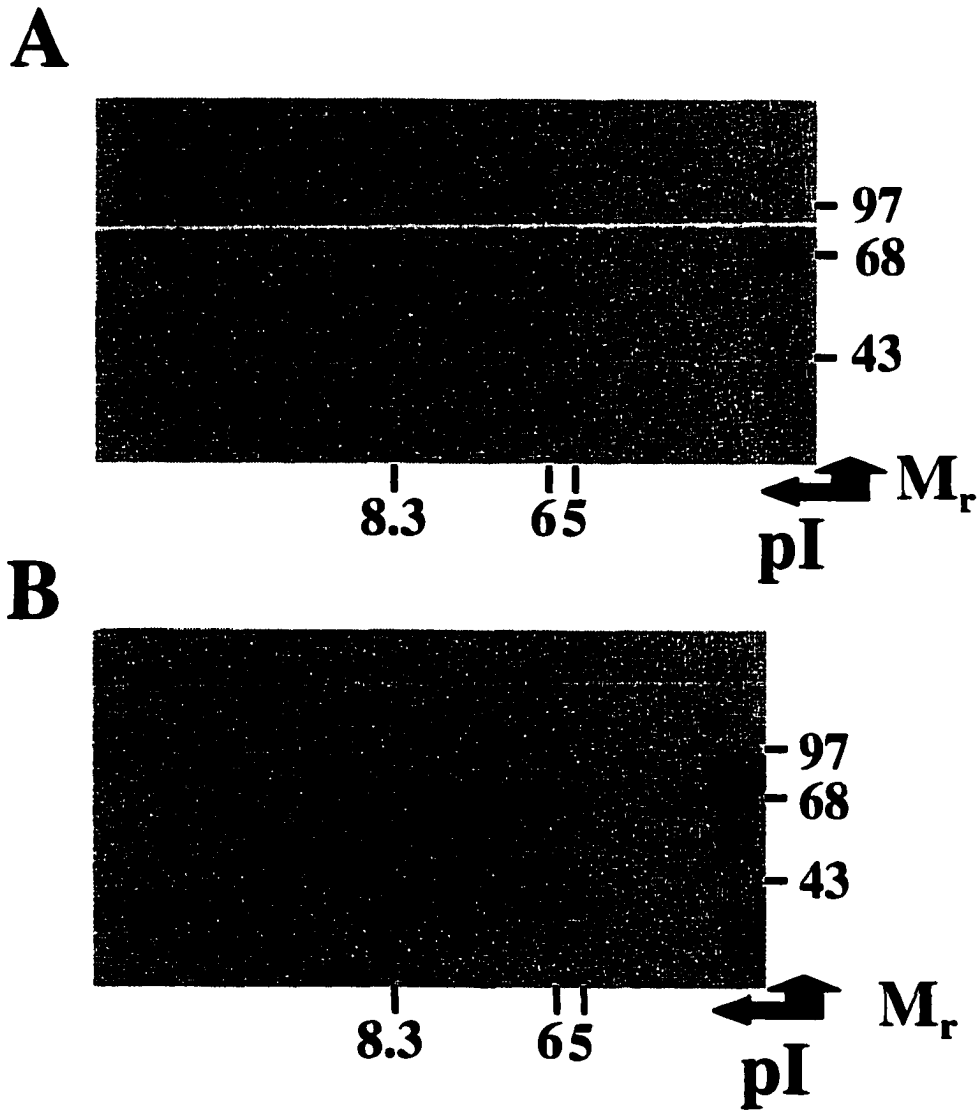


Fig. 7. **Analysis of *in vitro* translated tau by alkaline phosphatase digestion and two-dimensional electrophoresis (2D-NEPHGE).** [^{35}S]-radiolabeled tau expressed in reticulocyte lysate was incubated either with alkaline phosphatase (*A*) or reaction buffer (*B*) for 18 h at 37°C post-translation and subjected to two-dimensional electrophoresis. Molecular mass standards (kDa) are indicated at *right.* Internal isoelectric focusing standards indicated at *bottom.*

tau by proline-directed protein kinases alters its confirmation and impairs its mobility on SDS-polyacrylamide gels (104). In an attempt to identify a likely (Ser/ Thr) protein kinase responsible for the phosphorylation of tau in reticulocyte lysates, a panel of protein kinase inhibitors were tested for their ability to restore tau to its initial molecular mass on SDS-polyacrylamide gels (Fig. 5). Twenty-five milllimolar lithium chloride, an inhibitor of the GSK3β phosphorylation of tau (105), 1 μM wortmannin, an inhibitor of phosphatidyl inositol 3-kinase (PI-3 kinase) (106) and myosin light chain kinase (MLCK) (107), or 20 nM staurosporine, an inhibitor of MLCK (108), PKC (109), PKA (110), and protein kinase G (PKG) (111), were not able to restore tau to its initial molecular mass on SDS-polyacrylamide gels when added before or 2 h after the initiation of the *in vitro* transcription/translation reaction. Additionally, hypericin, an inhibitor of PKC (112), casein kinase II (113), and MAP kinase (114), or H-89, another PKA inhibitor (115), had no detectable effect on the gel mobility of *in vitro* translated tau (data not shown). Thus, GSK3β, PKA, PKC, MLCK, PI-3 kinase, casein kinase II, MAP kinase, and PKG are not likely involved in the endogenous phosphorylation of tau in reticulocyte lysates.

The *O*-GlcNAcylation of tau in the reticulocyte lysate was also investigated using the *in vitro* transcription/ translation system. While nucleoporin p62 bound wheat germ agglutinin (WGA)*Triticum vulgaris*, tau expressed in reticulocyte lysates in parallel did not bind, arguing that tau is not glycosylated by *O*-GlcNAc in this cell-free system. However, since tau becomes rapidly phosphorylated in reticulocyte lysates and glycosylation and phosphorylation sites appear to be similar on some proteins (116, 117) and tau, the glycosylation of tau may be inhibited in this assay. Tau is likely a better substrate for endogenous rabbit reticulocyte protein kinases; without their inhibition, the

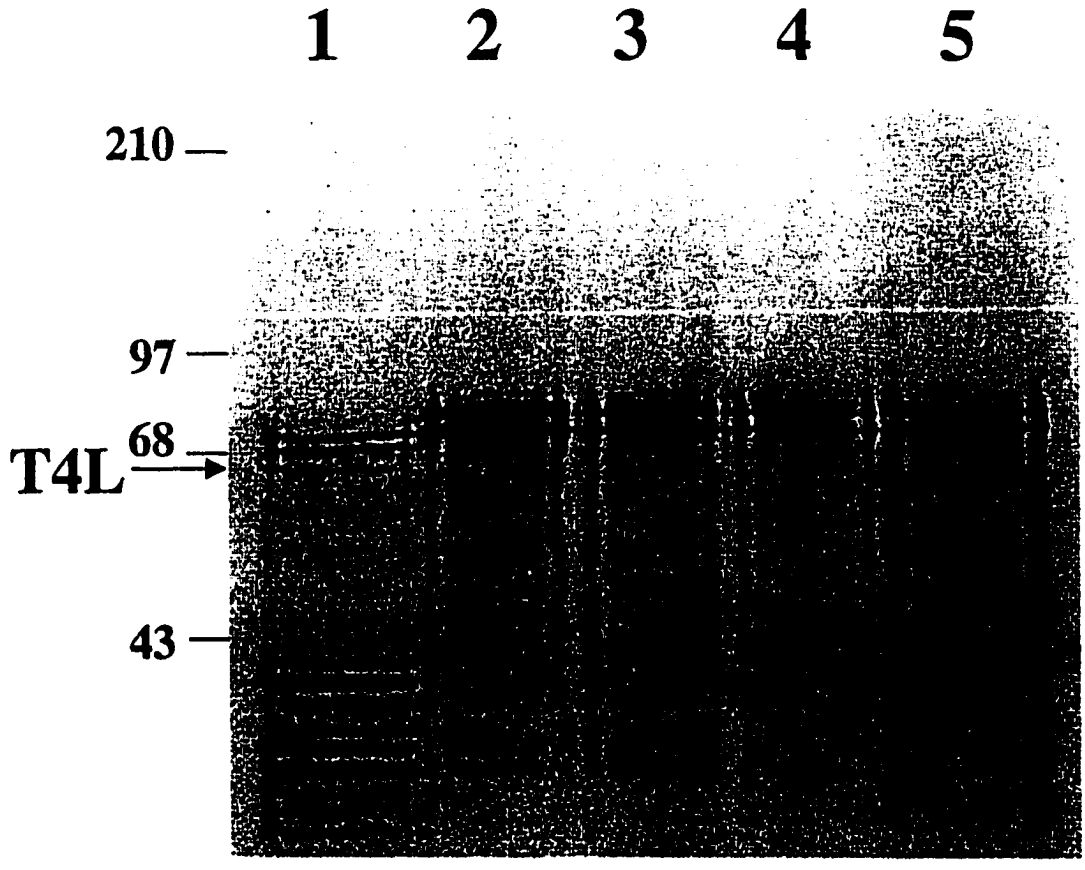


Fig. 8. **Effects of protein kinase inhibitors on gel mobility of *in vitro* translated tau.** *In vitro* translation of human recombinant tau dephosphorylated by alkaline phosphatase (*lane 1*). *lanes 2-5* represent the effects of protein kinase inhibitors on tau mobility when added post-translation: *2*, no inhibitor; *3*, 1 μM wortmannin; *4*, 20 nM staurosporine; *5*, 25 mM lithium chloride. *Arrow* indicates gel mobility of T4L purified from *E. coli*. Molecular mass standards (kDa) indicated at *left*.

glycosylation of tau may be significantly impaired. These results are consistent with the notion of a putative reciprocal relationship between the glycosylation and phosphorylation of tau.

Recently, investigators similarly demonstrated the phosphorylation of tau in reticulocye lysates upon addition of *Xenopus* mitotic extracts (118). Authors demonstrated that tau was phosphorylated at Thr-231, a residue which when phosphorylated promotes the interaction of prolyl isomerase Pin1, an essential mitotic regulatory protein. The phosphorylation of tau at Thr-231 has been documented for tau phosphorylated in vitro by cdc2 (119); cdk5; GSK3β (120); stress-activated protein kinases SAPK -1γ (121), SAPK3, and SAPK4 (122), and tau purified from PHFs (44). Phosphorylation of tau at Thr-231 causes an impaired migration on SDS-polyacrylamide gels and inhibits the ability of tau to bind microtubules *in vitro* (119, 120). Because the electrophoretic mobility of tau in these studies behaves similarly to tau when phosphorylated at Thr-231 and because the protein kinase inhibitors tested did not restore normal tau migration on gels, Thr-231 may be phosphorylated by a mitotic cdc2 kinase, cdk5, or SAPK endogenous to rabbit reticulocyte lysates. The *in vitro* transcription and translation of tau in this cell-free system may facilitate the identification of other protein kinases responsible for the phosphorylation of tau *in vivo* and lead to the development of potentially therapeutic inhibitors.

A Brain-specific *O*-GlcNAc Transferase?

Summary--O-GlcNAc transferase (OGT) is an enzyme that catalyzes the addition of *N*-acetylglucosamine from the donor sugar-nucleotide UDP-GlcNAc to Ser and Thr residues of nuclear and cytoplasmic proteins (75). Initial *in vitro O*-GlcNAcylation of

synthetic peptides suggests the existence of different OGT enzyme activities in rat liver and brain extracts. Rat tissues probed with an affinity-purified polyclonal antibody raised against the carboxyl-terminus of OGT revealed the presence of a brain-specific 120-kDa immunoreactive protein (p-120). p120 associates with rat brain microtubules through cycles of polymerization/depolymerization and co-sediments with microtubules assembled in the presence of guanosine 5'-triphosphate (GTP) and taxol. In-gel trypsin digestion of p120 immunoprecipitated from rat brain, followed by electrospray mass spectrometry, identified p120 as neuronal-specific kinesin heavy chain. The homology with OGT and the relevance to the glycosylation of different substrates are discussed.

Experimental Procedures--Materials--Male Sprague-Dawley retired breeder rats were obtained from Charles Rivers Laboratories. UDP-*N*-acetyl[6-^{3}H]glucosamine (50 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were of the highest commercial grade available.

Generation of Affinity-purified Antibody- All anti-OGT polyclonal antibodies used in this study were affinity purified as described (123) with the additional procedures. First, a carboxyl-terminus OGT antigen column was constructed. To generate antigen, a His-tagged C-terminal OGT construct was transformed into *E. coli* strain BL21DE3. LB-Ampicillin cultures were inoculated at 1% and grown until the OD at 600 nm was 0.4 to 0.6. Expression of the C-terminal construct was induced with 1 mM IPTG for 5 h. Cells were then pelleted at 7,000 x g for 10 min at 4°C; lysed in 8 M urea, 20 mM Hepes, pH 7.8, 0.5 M NaCl, 0.1% NonidetP-40, 5 mM imidazole, 1 mM PMSF, 10% glycerol, PICS 1, and PICS 2 at RT for 30 min; and sonicated 3 x 15 sec on power

setting 4 (Sonic Dismembranator 550, Fisher Scientific). Lysates were centrifuged at 27,000 x g for 20 min at 4°C. The supernatant (urea extract) was removed, and the pellets were dissolved in 6 M guanidine HCl, 20 mM Tris, pH 7.8, 0.1 M NaCl; vortexed; sonicated 3 x 15 sec; and centrifuged 27,000 x g for 20 min at 4°C (guanidine extract). Both urea and guanidine extracts were combined and passed through a 0.45-μm filter. The His-tagged OGT antigen was isolated from the extract by passage over 2 ml of Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) charged with 0.1 M $NiSO_4$ according to manufacturer's instructions. Antigen was eluted with urea extraction buffer in the presence of 0.5 M imidazole, dialyzed stepwise against 100 mM $NaHCO_3$, pH 8.3, 0.5 M NaCl, 2 mM DTT, and 0.5 M urea, with the initial concentration being 4 M urea. Antigen was then coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to manufacturer's instructions. Rabbit polyclonal antisera AL25 raised against full length p110 OGT and AL28 raised against the C-terminus of OGT were then passed over the antigen column, and the antibody was collected and stored as described (Harlow, Lane).

Isolation of Microtubules from Brain--Rat brain was homogenized with a polytron in MES buffer (100 mM MES, pH 6.6, 1 mM EGTA, 1 mM $MgSO_4$, PICS1, PICS-2, and 1 mM PMSF) at 4°C. The homogenate was sonicated 3 x 15 sec and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was subsequently centrifuged at 45,000 x g for 90 min at 4°C, yielding a tubulin-rich supernatant. The supernatant was divided and either was incubated on ice, warmed at 37°C, spiked with 1 mM GTP and warmed, or spiked with 20 μm taxol and 1 mM GTP and warmed. All fractions were

incubated at 37°C for 25 min to allow microtubules to polymerize, added over an equal volume of 10% sucrose/ MES buffer, and centrifuged at 45,000 x g at 25°C for 30 min. Pellets were solubilized in MES buffer and 0.5% of samples were analyzed by Western blot for the presence of OGT, vinculin, MAP2, or tubulin.

Microtubule Cycling--The polymerization/depolymerization of microtubules was performed as described previously (124) with some minor modifications. Fresh rat brains were homogenized with a polytron in (1:1) w:vol in (P) buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$) + PICS 1 + PICS 2, 1 mM PMSF, and 0.1 mM GTP. The homogenate was centrifuged at 7,500 x g for 1 h at 4°C. The low-speed supernatant (LSS) was recentrifuged at 35,000 x g for 45 min at 4°C. The high-speed supernatant (HSS) was brought to 25% glycerol and 1 mM GTP and incubated for 45 min in a 37°C water bath. Microtubules (P1) were pelleted at 35,000 x g for 2 h at 25°C; resuspended in (P) buffer + 0.1 mM GTP, PICS 1, PICS 2, and 1 mM PMSF; transferred to a teflon homogenizer, and thoroughly homogenized. The (S1) supernatant was stored at -80°C until analyzed. The solubilized microtubules were then depolymerized on ice for 35 min and recentrifuged at 35,000 x g for 35 min at 4°C. The supernatant was spiked with 1 mM GTP and incubated at 37°C for 45 min. The second cycle of polymerized microtubules (P2) were isolated by centrifugation at 35,000 x g for 45 min at 25°C, and the (S2) supernatant was collected and stored at -80°C until further analysis.

Western Blot Analysis of Rat Tissues--Rat tissues were homogenized in 0.1 M PIPES, pH 6.9, 5 mM MgCl2, 1 mM EGTA, PICS1, PICS2, and 1 mM PMSF with a polytron homogenizer on ice. Homogenates were sonicated 3 x 15 sec and further centrifuged at 10,000 x g for 10 min at 4°C. LSSs were recentrifuged at 120,000 x g for 1 h at 4°C. HSSs were assayed for protein content, aliquotted, and stored at -80°C. Samples were electrophoresed on SDS-polyacrylamide gels and transferred to PVDF in 25 mM Tris and 190 mM glycine. PVDF membranes were preblocked for 1 h at room temperature in blotting buffer (5% milk in 10 mM Tris, pH 8.0, 100 mM NaCl, and 0.05% Tween-20; TBST) prior to probing with AL28 (1:5,000), anti-tubulin (Sigma) (1:30,000), or anti-MAP2 (Sigma) (1:20,000) in blotting buffer at 4°C overnight. Primary antibodies were detected with either donkey anti-rabbit-HRP (1:10,000) (Amersham Pharmacia Biotech) or sheep anti-mouse-HRP (1: 10,000) (Amersham Pharmacia Biotech) in blotting buffer, followed by the ECL Plus (Amersham Pharmacia Biotech) Western blotting detection system.

Immunoprecipitation--Rat liver or brain homogenates (*HSS*) were incubated with 4 mg of affinity-purified AL25 antibody or equivalent amounts of pre-immune serum for 3 h at 4°C. The immune complexes were then precipitated with protein A-sepharose CL4-B (Amersham Pharmacia Biotech) in TBST, 0.1% Tween-20. The precipitates were washed extensively with TBST, boiled in Laemlli buffer (95), resolved on SDS-polyacrylamide gels and visualized by silver staining.

OGT Assay- The peptide substrates used for the assay, YSPSDSTST and PGGSTPVSSANMM, were synthesized by standard F-moc chemistry on a Perseptive Bio-

systems synthesizer (Farmington, MA), purified by RP-HPLC, and confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. OGT assays were performed as previously described (75).

Results and Discussion- An initial study comparing the enzymatic activities of OGT from brain versus liver against standard synthetic peptides indicates that rat brain contains more OGT activity than rat liver. A peptide from casein kinase II, PGGSTPVSSANMM, and standard peptide, YSDPSTST, were used in the assay of desalted 75% ammonium sulfate precipitates. Figure 9*A* demonstrates the in-creased [^{3}H]GlcNAc incorporation into the casein kinase II peptide from rat brain homogenate versus liver, indicating more OGT activity/mg protein in brain. However, when the data are plotted as a percentage of the standard peptide, YSDPSTST, brain also appears to contain a higher specific activity toward casein kinase II than liver (Fig. 9*B*), suggesting a different OGT enzyme activity in brain.

In order to evaluate the existence of a putative brain-specific OGT, an affinity-purified polyclonal rabbit antibody directed against the C-terminus of the enzyme (AL-28) was generated. The C-terminus was chosen as antigen because this region likely contains the enzyme's catalytic site (58, 59). Western blot analysis with AL28 illustrates the presence of OGT in numerous rat tissues (Fig. 10). All tissues contain the p110 subunit of the enzyme, whereas the p78 subunit is observed in spleen and kidney. As previously reported (58, 59), OGT is much more abundant in brain and thymus than in liver, kidney and spleen. However, there exists a previously unidentified 120-kDa immunoreactive polypeptide (p120) specific to brain.

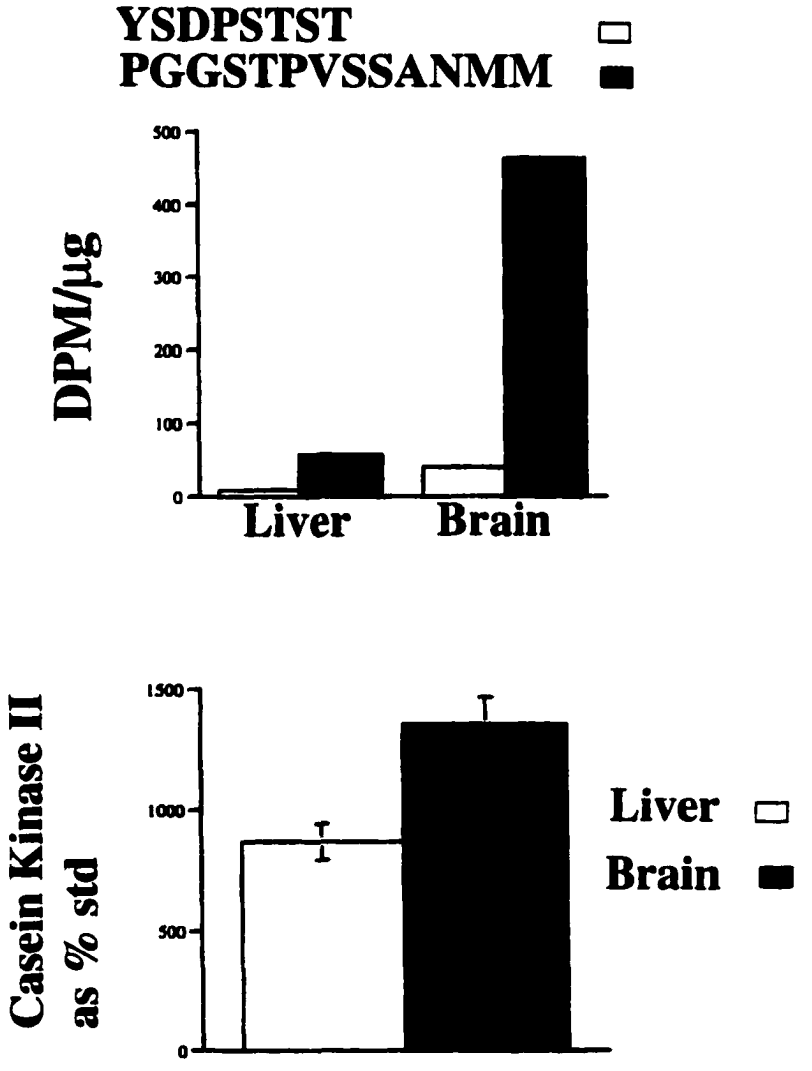


Fig. 9. ***O*-GlcNAcylation of synthetic peptides by rat liver and brain extracts.** *A*, 45% Ammonium sulfate precipitates of rat brain and liver were desalted and assayed for the ability to incorporate [^{3}H]GlcNAc from the donor sugar nucleotide, UDP-[^{3}H]-GlcNAc, into a standard synthetic peptide, YSDPSTST, or a peptide from casein kinase II, PGGSTPVSSANMM. *B*, comparing the specific activities of brain vs. liver, data from the *O*-GlcNAcylation of the casein kinase II peptide were plotted as a percentage of standard.

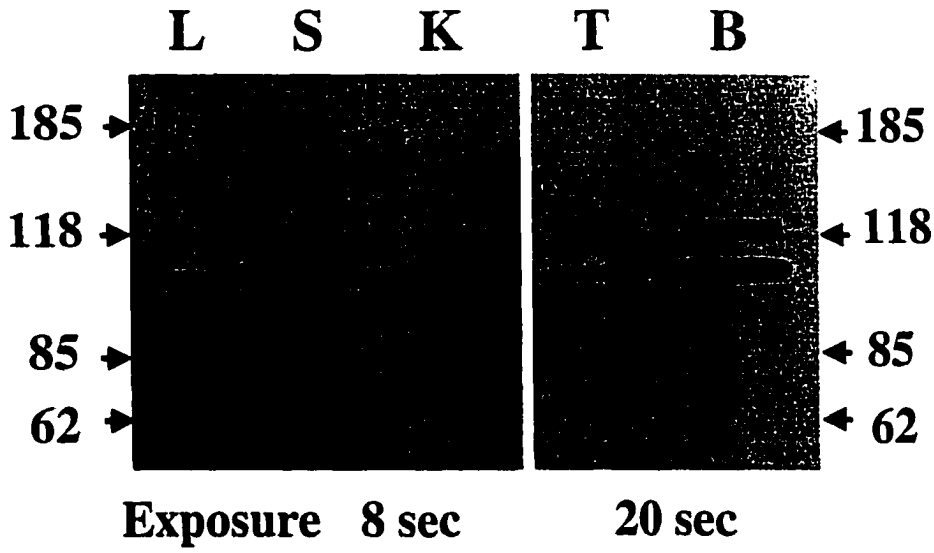


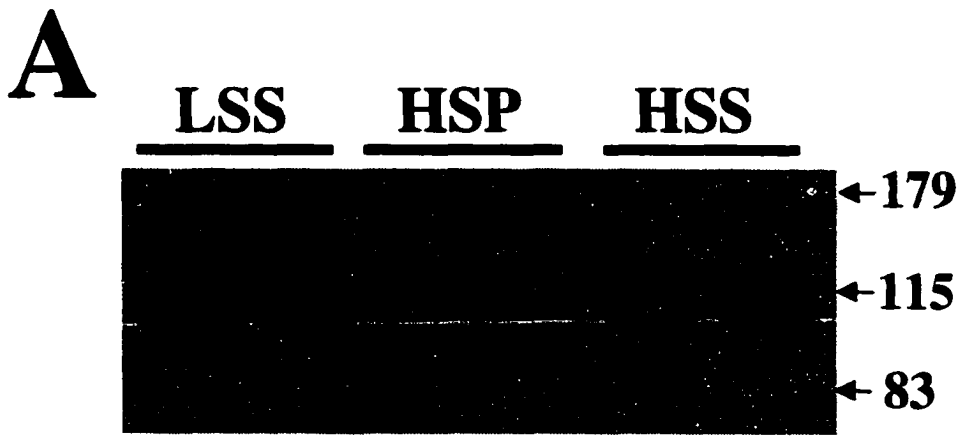
Fig. 10. **Western blot of rat tissues with antigen-purified AL28 rabbit polyclonal antibody.** 25 μg of rat tissue homogenate from (*L*) liver, (*S*) spleen, (*K*) kidney; (*T*) thymus, and (*B*) brain were resolved on a 10% SDS-polyacrylamide gel, transferred to PVDF, and probed with antigen-purified AL28 rabbit polyclonal antibody. Positions of molecular mass standards (kDa) are indicated in the margin and exposure times for enhanced chemiluminescence are indicated at *bottom*.

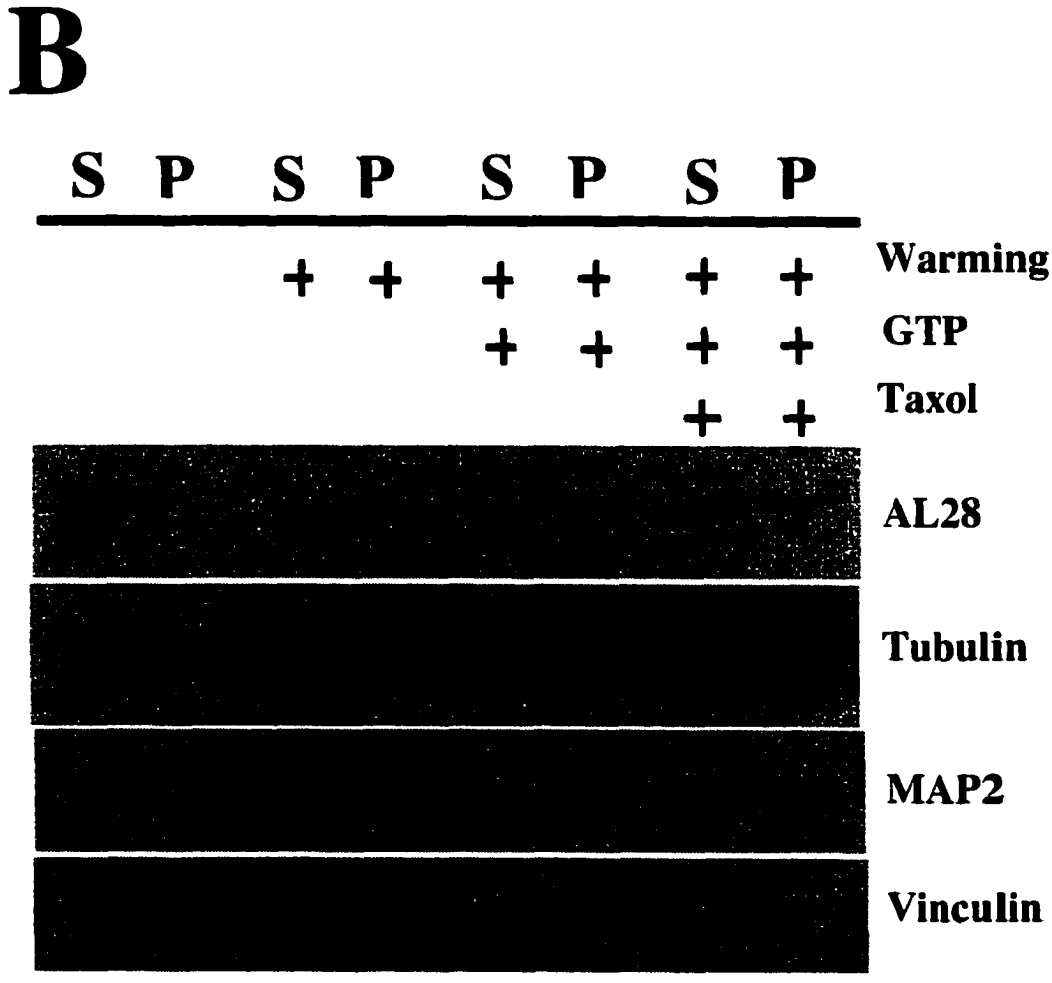
Several MAPs including MAP1, MAP2, MAP4, and tau, are all extensively modified by *O*-GlcNAc (66, 125). In addition, protein phosphorylation and *O*-GlcNAc-ylation appear to be reciprocally related within the cytoskeleton (126). Since enzymes involved in protein phosphorylation, including MAP kinase (erk2) (127, 128), cdc2 kinase (129), and protein phosphatase 1 (130), interact with micro-tubules and their associated proteins, the putative association between OGT and rat brain microtubules was investigated.

The incubation of tubulin-rich fractions from rat brain with either GTP or GTP and taxol promotes the polymerization of αβ-tubulin dimers into microtubules. Micro-tubules can be subsequently pelleted by centrifugation, and their interacting proteins detected by Western blotting. After obtaining an HSS from rat brain extract (Fig. 11*A*), only two major AL28 immunoreactive species are present, p110 OGT and p120. This HSS fraction was either incubated on ice, was warmed, or was warmed in the presence of GTP or of GTP and taxol. Upon analysis of microtubule-containing fractions by Western blot analysis, the 110-kDa subunit of OGT did not co-sediment with micro-tubules assembled in the presence of GTP or taxol (Fig. 11*B*). However, a significant fraction of p120 did associate with microtubules in this assay, arguing that they interact. The specificity of the interaction is demonstrated by the positive control, MAP2, which co-sediments with microtubules, and the negative control, vinculin, an actin-associated protein that does not sediment with microtubules (Fig. 11*B*).

To verify the interaction between p120 and rat brain microtubules, a microtubule polymerization/depolymerization assay was employed. Tubulin spontaneously poly-merizes into microtubules at 37°C in the presence of 1 mM GTP and, conversely, de-polymerizes on ice. These biochemical properties were utilized as a method to deter-

Fig. 11. **The 120-kDa anti-OGT immunoreactive protein co-sediments with microtubules.** *A*, rat brains were homogenized; fractionated into a low-speed supernatant (*LSS*), a high-speed pellet (*HSP*), and a high-speed supernatant (*HSS*); electrophoresed on a 6% SDS-polyacrylamide; transferred to PVDF; and probed with the antigen-purified polyclonal antibody AL28. Positions of molecular mass standards (kDa) are indicated at *right*. *B*, the HSS fraction was either incubated on ice, was warmed to 37°C, or was warmed in the presence of GTP or of GTP and taxol to facilitate the polymerization of microtubules. Samples were then centrifuged through a 10% sucrose cushion, and fractions of supernatant (*S*) and pellet (*P*) were analyzed by Western blot for the presence of OGT, tubulin, MAP2, or vinculin.





mine if p110 OGT or p120 co-assembled with rat brain microtubules. After one cycle of microtubule polymerization, p120 associates with microtubuless (P1), whereas p110 OGT does not (S1) (Fig. 12). The amount of p120 that associates is approximately half the p120 in the HSS fraction. The ability of p120 to assemble with microtubules is further strongly supported by its association through a second cycle of microtubule polymerization (P2), indicating p120 interacts with rat brain microtubules.

In order to determine if p120 was related to OGT at the primary sequence level, proteins were immunoprecipitated, digested with trypsin, and sequenced by LC/ESMS. After immunoprecipitation with antigen-purified AL25 rabbit polyclonal antibody, both p110 and p120 were detected by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 5). The specificity of the precipitation was controlled by a preimmune control (*lane 2*) (Fig. 13). In-gel trypsin digestion, followed by LC/ESMS sequencing of peptides from the 110-kDa band, confirmed its identity as the 110-kDa polypeptide OGT. However, seven tryptic peptides from the 120-kDa band identified this protein as the heavy chain of neuronal specific kinesin. Kinesin is a microtubule motor, explaining its ob-served association with rat brain microtubules. Unfortunately, kinesin and OGT are not related at the polypeptide level, but rather likely contain homologous regions of tertiary structure responsible for the cross-reactivity observed with OGT antibodies.

OGT is an enzyme that catalyzes the addition of *N*-acetylglucosamine from the donor sugar-nucleotide UDP-GlcNAc to Ser and Thr residues of nuclear and cytoplasmic proteins. OGT was originally purified from rat liver and was subsequently cloned from rat and human cDNA libraries. Recently, the gene was located on the X chromosome at Xq13, a region associated with neurological disease (131). Embryonic stem-cell knockouts are not viable, arguing that the glycosylation of proteins by *O*-

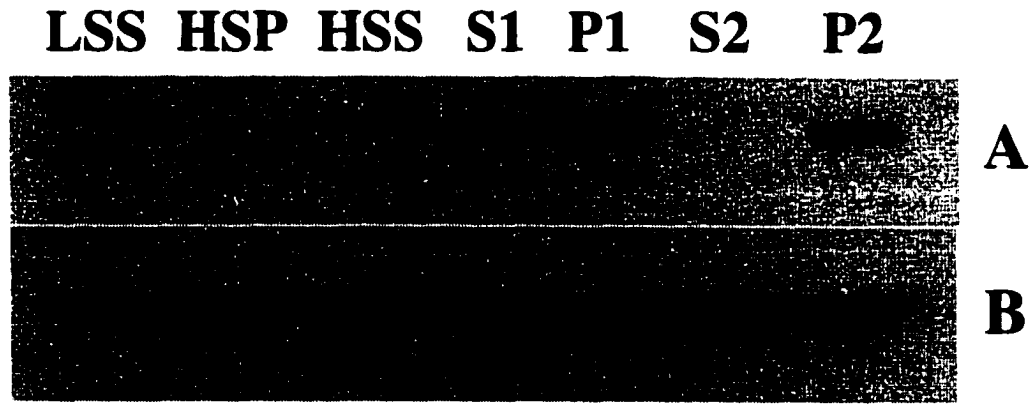
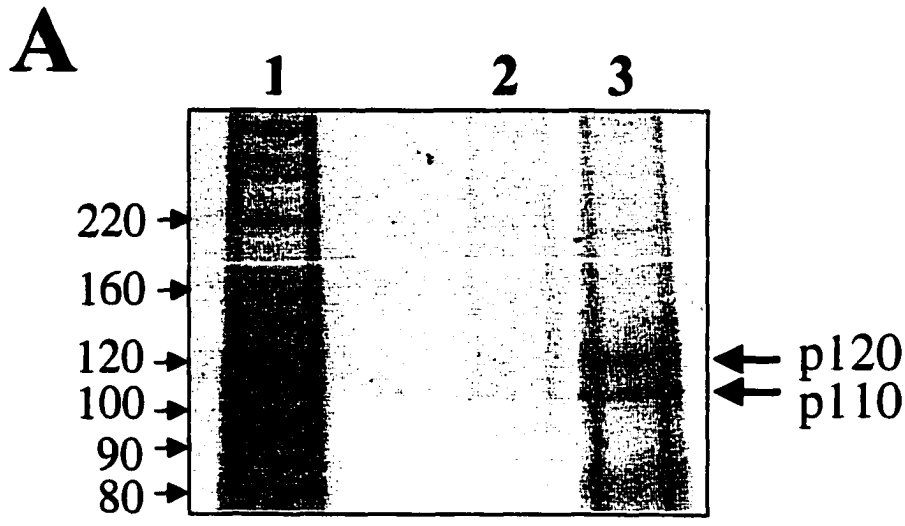


Fig. 12. **Western blot of cycling of rat brain microtubules.** Rat brain homogenates were fractionated into a low-speed supernatant (*LSS*), high-speed pellet (*HSP*), and high-speed supernatant (*HSS*). Microtubules (*P1*) and (*P2*) were pelleted in the presence of 25% glycerol and 1 mM GTP. *A*, 25 μg of each fraction were resolved on a 6% SDS-polyacrylamide gel, transferred to PVDF, and probed with AL28 antibody. *B*, 5 μg of each fraction were resolved on 8% SDS-polyacrylamide gels, transferred to PVDF, and probed with anti-tubulin mAb.



B

Peptides Sequenced from Neuronal Specific Kinesin (117,301)

TGAEGAVLDEAK
SLSALGNVISALAEGTK
LYISK
ELQTLHNLR
ISFLENNLEQLTK
YQQEVDRIK

Fig. 13. **Immunoprecipitation and identification of p120.** *A*, silver-stained 10% SDS-polyacrylamide gel of rat brain extract *(lane 1)*, antigen-purified AL25 immunoprecipitated complexes with protein-A (*lane 3*), or pre-immune control (*lane 2*). Molecular mass standards (kDa) are indicated at *left*. *B*, Tryptic peptides from p120 analyzed by LC/ESMS identify p120 as neuronal specific kinesin heavy chain.

GlcNAc is essential for life at the single-cell level. Structurally, the *O*-GlcNAc transferase is different from any previously identified glycosyltransferase. Within the amino-terminus are several tandem tetratricopeptide repeats (TPR) domains that are suggested to play a role in mediating inter- and intramolecular protein-protein interactions. The TPR domains have been demonstrated to promote the multimerization of the enzyme, as well as affect the binding affinity of the donor sugar nucleotide UDP-GlcNAc (61). Although initial data with synthetic peptides argue the presence of tissue-specific related enzyme activity, the existence of a brain-specific OGT is not supported by these findings. Results from these studies suggests a single polypeptide enzyme in all tissues examined and support the notion of TPR-associated factor-mediated control of substrate specificity as previously hypothesized (58).

CONCLUSIONS

Tau is the predominant protein component of the paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) described in the neuropathology of Alzheimer's disease (AD). Tau purified from PHFs (PHF-tau) is abnormally hyperphosphorylated, no longer binds microtubules, and self-associates into a precipitated proteinaceous form that likely contributes to neuron death. As expected, the phosphorylation of tau and metabolic factors that regulate this post-translational modification are well studied. However, it is still unclear whether phosphorylation is responsible for the formation of PHFs, as other modifications such as ubiquitination and glycation have been demonstrated to affect the post-translational processing of tau. Nevertheless, phosphorylation clearly affects the function of tau, and other modifications which might affect tau phosphorylation could significantly alter the post-translational processing of tau and contribute to the deposition of pathological lesions.

Another post-translational modification, *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), has been described for numerous nuclear and cytoskeletal proteins. This dynamic modification is found on multimerizing phosphoproteins and has been suggested to play a role in protein-protein complex formation, protein phosphorylation, and protein stability. Recently, same-site occupancy of phosphorylation and *O*-GlcNAcylation has been described for the corresponding hydroxyl of threonine (Thr)-58 on the c-Myc oncoprotein. As well, the carboxyl-terminal domain of RNA polymerase II is either distinctly *O*-GlcNAcylated or phosphorylated. Accordingly, a reciprocal relationship between *O*-Glc-

NAcylation and phosphorylation has been suggested on some proteins. For example, the transcription factor Sp1 becomes hypophosphorylated when HT29 cells are treated with *O*-(2-acetomido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), an inhibitor of *O*-GlcNAc removal. Likewise, the *O*-GlcNAcylation of cytoskeletal proteins in cultured neurons changes inversely to that of protein phosphorylation. These studies describe the investigation into the glycosylation of tau by this unique posttranslational modification.

While probing purified bovine tau with β(1,4)galactosyltransferase (β(1,4)GT) in the presence of uridine diphosphate (UDP)-[^{3}H]galactose, all bovine tau isoforms were radiolabeled, indicating that tau is glycosylated. Tau was also demonstrated to be glycosylated by the detection of terminal GlcNAc residues utilizing the lectin succinylated wheat germ agglutinin (sWGA). For the characterization of the exact nature of the [^{3}H]-galactosylated sugars on tau, radiolabeled proteins were first digested with P*N*Gase F, an enzyme that specifically cleaves *N*-linked carbohydrates at the peptide backbone. Radiolabeled carbohydrates on tau were resistant to P*N*Gase F digestion, indicating they were not *N* linked. They were, however, labile to alkaline β-elimination, suggesting that tau contained *O*-linked sugars. Gel-filtration analysis followed by high-pH anion exchange chromatography of β-eliminated radiolabeled sugars identified the carbohydrates as single *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) moieties, indicating tau is modified by *O*-GlcNAc.

To determine if any differences existed in the glycosylation of the separate individual isoforms, [^{3}H]galactosylated tau proteins were separated into their individual isoforms by SDS-polyacrylamide gel electrophoresis, followed by in-gel trypsin digestion

and reverse-phase high performance liquid chromatography (RP-HPLC) of the resulting [^{3}H]galactosylated glycopeptides. Resolution of the resulting tau tryptic glycopeptide profiles indicated that all bovine tau isoforms were glycosylated with approximately over 12 sites of glycosylation with some minor stoichiometric differences at individual sites. However, when stoichiometric analysis was performed, it was determined that tau contains on average ~4 mol GlcNAc/mol of protein, suggesting substoichiometric site occupancy.

For the identification of individual sites of *O*-GlcNAc attachment, tryptic tau peptides were labeled by [^{3}H]galactosylation using galactosyltransferase, and the radiolabeled glycopeptides were purified by several rounds of RP-HPLC. The subsequent gas-phase sequencing of two major radioactive peaks identified the peptide sequences IGSTENLK and SKIGSTENLK. Both sequences lie within the first microtubule-binding repeat of tau and contain the amino acid Ser-262, a PHF-tau phosphorylation site which virtually abolishes tau binding to microtubules when phosphorylated *in vitro*. Glycosylation of this region was confirmed by *O*-GlcNAcylation of a synthetic peptide, CVSKIGSTENLKHQ, with rat brain extract. The glycosylation of this region, either at Ser-262 or at Thr-263, may prevent tau phosphorylation at Ser-262, therefore enabling tau to bind and stabilize microtubules.

Another site of tau *O*-GlcNAcylation was identified by the *in vitro O*-GlcNAcylation of a peptide from the carboxy-terminus of tau, SPVVSGDTSPR. Manual Edman degradation of the [^{3}H]GlcNAcylated peptide identified the site of attachment as Ser-400, a GSK3β site of phosphorylation and PHF-tau phosphorylation site unique to Alzheimer's brain. *O*-GlcNAc at Ser-400 would likely prevent the reciprocal phosphorylation at Ser-400 by GSK3β. Further evidence supporting the exclusive *O*-GlcNAcylation or phos-

phorylation of tau is the finding that tau is phosphorylated but not *O*-GlcNAcylated when expressed in rabbit reticulocyte lysates. The same-site occupancy of *O*-GlcNAcylation and phosphorylation of tau observed in these studies argue a role for *O*-GlcNAc in possibly regulating the phosphorylation of tau.

Finally, an investigation into the existence of a putative brain-specific *O*-GlcNAc transferase (OGT) was initiated with the use of an antigen-purified rabbit polyclonal antibody. Western blots of rat tissues identified a 120-kDa immunoreactive protein (p120) that associated with rat brain microtubules through cycles of polymerization/depolymerization, as well as co-sedimented with microtubules. Immunoprecipitation of p120, followed by in-gel trypsinization and subsequent analysis of the resulting tryptic peptides, identified p120 as neuronal specific kinesin heavy chain. Kinesin is a microtubule motor protein, explaining its observed association with microtubules. The lack of amino acid homology with OGT suggests that kinesin shares some three-dimensional conformation with OGT, explaining its cross-reactivity with anti-OGT antibodies.

To evaluate more carefully the relationship between the *O*-GlcNAcylation and phosphorylation of tau, several approaches might be taken. One possibility could include a comparative mass profile analysis of tau from AD and normal brains. However, the harsh chemical methods (8, 9) used to isolate the highly insoluble PHFs would likely remove any endogenous glycans on tau and, ultimately, make data interpretation difficult. Additionally, due to the fact that *O*-GlcNAc is highly labile to some ionization techniques (132), the successful use of electrospray mass spectrometry for the detection of *O*-GlcNAc may sometimes be peptide dependent and, ultimately, fraught with difficulties.

Another possible approach to determining the relationship between tau *O*-GlcNAcylation and phosphorylation might include the use of phosphorylation site-specific

antibodies to tau. Investigators have previously demonstrated the site-specific regulation of tau phosphorylation in the developing rat brain (133). Tau phosphorylation at sites Ser-202, Thr-205, Ser-396, and Ser-404 is elevated from embryonic to postnatal day 21 in the developing rat. Phosphorylation of tau at these residues subsequently decreases in the adult brain. To determine the relationship between tau *O*-GlcNAcylation and phosphorylation in this model, one could confirm the phosphorylation of tau with these antibodies and simultaneously probe for *O*-GlcNAc with Gal β(1-4) galactosyltransferase in the presence of [^{3}H]-UDP-Gal or GlcNAc specific lectins. These phos-phorylation site-dependent tau antibodies might also be used to isolate tau that is phosphorylated and then thereafter determine its state of *O*-GlcNAcylation.

To address more carefully the role of *O*-GlcNAc at Ser-400 on the phosphorylation of tau, a synthetic glycopeptide corresponding to this C-terminal region and based upon the *in vitro* site-mapping data could be synthesized. The resulting glycopeptide could be incubated with recombinant glycogen synthase kinase 3β (GSK3β), the enzyme known to phosphorylate tau on serine (Ser)-400 *in vitro*, in order to evaluate *O*-GlcNAc's effect on phosphorylation. Also, one could similarly determine the effect of phosphate at Ser-400 on the *in vitro* *O*-GlcNAcylation of a similar peptide by OGT.

Ultimately, these studies identify tau as a member of the growing list of *O*-GlcNAcylated cytoskeletal proteins. The high degree of glycosylation of bovine tau indicates a possible structural role for these glycoconjugates on tau. The findings that tau is *O*-GlcNAcylated within the first microtubule-binding domain and that other microtubule-associated proteins (MAPs) including MAP1, MAP2, and MAP4, are also *O*-GlcNAcylated suggest a role for *O*-GlcNAc in mediating the interactions of the MAPs with tubulin. The discovery that tau is glycosylated at Ser-400, a PHF-tau phosphorylation

site, demonstrates same-site occupancy and is consistent with the hypothesis of a reciprocal nature between tau phosphorylation and glycosylation. The implications of possible *O*-GlcNAcylation and phosphorylation reciprocity on tau are many. Because phosphorylation affects the degradation of tau and tau's interactions with microtubules and is diagnostic for the PHFs observed in several neurodegenerative disorders, *O*-GlcNAc may play an important role in the normal or pathological post-translational processing of tau.

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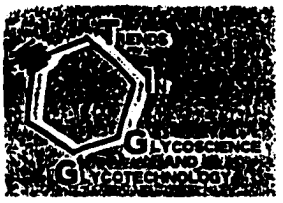

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June 13, 2000

Mr. C. Shane Arnold
213 West 29th Street Apt. 2
Baltimore, MD 21211
USA

Dear Mr. Arnold,

FCCA agrees that you reformat and publish in microfilm the article entitled "Dynamic Cytoskeletal Glycosylation and Neurodegenerative Disease", contributed by yourself and Dr. Gerald Hart, published in TIGG vol. 11, No. 62. 355-370, 1999. FCCA also agrees to save and hold harmless University Microfilms International from damages that may arise from copyright violations.

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APPENDIX B

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June 13, 2000

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Baltimore, MD 21211

Dear Arnold:

Please note your request for copyright permission has been granted for the following:

Volume:	Page:	Year
271	28641-28744	1996

'The Microtubule-associated protein tau is extensively modified by O-linked...'

For future requests, we can be contacted by e-mail at jbc@asbmb.faseb.org. If you have any questions, please contact us at the above number.

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Joan Gilbert

For the copyright owner
THE AMERICAN SOCIETY FOR BIOCHEMISTRY
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APPENDIX C

INSTITUTIONAL ANIMAL CARE AND USE APPROVAL FORMS

JOHNS HOPKINS
UNIVERSITY

Animal Care and Use Committees
720 Rutland Avenue
Baltimore MD 21205
(410) 955-3273 / FAX (410) 550-5068

Date: 04/10/98

MEMORANDUM TO: Dr. Gerald W Hart

FROM : Animal Care and Use Committee

RE Protocol: MO97M209
Title : Role of dynamic o-clycosylation of cytoskeletal proteins in neurodegenerative disease of aging

EXPIRATION DATE: 06/26/98

Your animal research protocol will expire in 60 days. If you intend to continue this project, you must submit an ANIMAL RESEARCH PROTOCOL RENEWAL FORM ng later than ONE MONTH prior to the expiration date. After the expiration date indicated above, you will not be able to use this number for animal ordering or for research grant applications. Renewal applications WILL NOT be accepted after the expiration date. If the protocol has expired, a new PROTOCOL REVIEW FORM must be submitted for Committee review. If you do not plan to continue the studies, indicate this at the bottom of the memorandum and return it to the Animal Care and Use Committee office.

Send your renewal application at least ONE MONTH prior to the expiration date to the Animal Care and Use Committee for your School. YOU WILL RECEIVE ONLY ONE REMINDER. PLEASE RESPOND PROMPTLY.

School of Medicine
Ms. Lisa Wetzelberger
c/o Division of Comparative Medicine
459 Ross

School of Hygiene & Public Health
Ms. Leslie Graf
Office of Research Subjects
School of Hygiene and Public Health, Rm. 1606

School of Arts & Sciences:
Ms. Donna Bragg
Mudd Hall, Room 144

This is a 1 year Renewal.

____ Study will not be continued

Gerald W Hart Signature of Principal Investigator

JOHNS HOPKINS UNIVERSITY ANIMAL CARE AND USE COMMITTEE
ANIMAL RESEARCH PROTOCOL RENEWAL REQUEST

Rec'd: _____
O Log O Database
For ACUC Use Only

Protocol #: M097M209 Last Approval Date: 6/26/97

Principal Investigator: Gerald W Hart Department: Biol. Chem

E-Mail Address: gwhart@bs.jhmi.edu Telephone: 614-5993

JHU Address: ~~401~~ 401 Hunterian, Sch of Med. Fax No: 614-8804

Coinvestigators: C. Shane Arnold, Sai Iyer, Glendon Parker, Ph.D

Title of Protocol: Role of Dynamic O-Glycosylation of Cytoskeletal Proteins in Neurodegeneration Diseases of Aging

Species: Mice Approximate Number Needed Per Year <100 no change
(Justify any change in number from original protocol)

ANSWER ALL OF THE FOLLOWING QUESTIONS:

Yes	No	
_	X	Is there a substantial departure from the previously approved research plan? If YES, complete a new Protocol Review Form describing the changes and submit the form with this application.
_	X	Are there minor changes proposed for the research plan? If YES, provide details which can be treated as an amendment in an attachment.
_	X	Have any unexpected complications or findings been noted? If YES, describe these in an attachment.
_	X	Has exposure to any source of radiation or the use of radioactive materials changed from that indicated in the original protocol? If YES, describe _____
_	X	Have the organisms pathogenic to humans or animals changed from those indicated in the original protocol? If YES, describe _____
_	X	Has the use of recombinant DNA or transgenic animals changed from those indicated in the original protocol? If YES, describe _____
_	X	Have the carcinogenic, highly toxic or hazardous chemicals changed from those indicated in the original protocol? If YES, describe _____
_	X	Have the anesthetics or analgesics changed? If YES, describe _____
X	_	Staff Changes (School of Hygiene staff must sign protocol renewal form). Indicate changes in staff who will independently use this protocol. Include the names of faculty, fellows, graduate students and technicians in your Progess Report on the back of this sheet. INFORMATION REGARDING QUALIFICATIONS AND TRAINING MUST BE PROVIDED. CV's ARE NOT ACCEPTABLE.

Arnold & Iyer were on prior approved proposal.
Dr. Glendon Parker has received extensive training in the use of animals during his ph.D. training in Australia and at the univ. of Maryland.

<<<< OVER >>>>

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Christopher S. Arnold

Major Subject Biochemistry

Title of Dissertation Dynamic Glycosylation of the Microtubule-Associated Protein Tau and Neurodegenerative Disease

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

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