Neurofibrillary tangles (NFTs) are found in a wide range of neurodegenerative disorders, including Alzheimer’s disease. The major component of NFTs is aberrantly hyperphosphorylated microtubule-associated protein tau. Because appropriate in vivo models have been lacking, the role of tau phosphorylation in NFTs formation has remained elusive. Here, we describe a new model in which adenovirus-mediated gene expression of tau, ΔMEKK, JNK3, and GSK-3β in COS-7 cells produces most of the pathological phosphorylation epitopes of tau including AT100. Furthermore, this co-expression resulted in the formation of tau aggregates having short fibrils that were detergent-insoluble and Thioflavin-S-reactive. These results suggest that aberrant tau phosphorylation by the combination of these kinases may be involved in “pretangle,” oligomeric tau fibril formation in vivo.

Filamentous tau aggregates is the major component of neurofibrillary tangles (NFTs)⁴ (1), the most common neuropathological hallmark in several neurodegenerative disorders, including Alzheimer’s disease (AD). Discovery of the molecular mechanisms of NFT formation may provide more direct insight into the process of neurodegeneration in AD. NFTs consist of highly phosphorylated microtubule-associated protein tau that assembles to form fibrils with β-sheet structures within the cell body and dendrites of neurons (2, 3). Several in vitro studies reveal that the repeat domain of tau aggregates more readily than full-length tau (4, 5) and forms the core of tau fibrils in AD (6). Moreover, this aggregate formation is enhanced by the presence of a polyanion such as heparin (7, 8) or by RNA (9) or fatty acids (10) in the absence of tau phosphorylation. However, these in vitro conditions may not be relevant to the mechanism underlying the formation of NFTs, because tau is always aberrantly hyperphosphorylated in AD. Therefore, it would seem necessary to consider the role of hyperphosphorylation of tau in the abnormal aggregation of filamentous tau.

The assembly of phosphorylated tau was also observed during the presence of 4-hydroxy-2-nonenal (11), a lipid peroxidation by-product of oxidative stress. Increased oxidative stress is reported to occur in AD (12–15). Interestingly, phospho-tau immunoreactive neurons are also stained positively with 8-hydroxy-2′-deoxyguanosine, another marker for oxidative stress (16, 17). These results suggest that oxidative stress may, in part, trigger the formation of NFTs. If oxidative stress does participate in NFTs formation in AD, such stress can lead to the activation of kinases that phosphorylate tau and stimulate NFT formation. One such candidate kinase is stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), a member of the mitogen-activated protein kinase family that is activated by several kinase cascades. Recent studies, employing antibodies against paired helical filaments (PHFs), have reported that activated phospho-JNK co-localized in neurons displaying PHF immunoreactivities (18, 19). Phospho-JNK was also shown to translocate from the nucleus to the cytoplasm in NFT-bearing neurons (19). Furthermore, JNK has been shown to phosphorylate tau at Ser322 (20), a site that is specifically phosphorylated in AD brains (21).

To understand the role of tau phosphorylation in NFT formation, we sought to develop a model that could reproduce the aberrant tau phosphorylation, including phosphorylation of Thr231 and Ser394, key sites commonly referred to as the AT100 epitope. The AT100 epitope appears to be more specific for Alzheimer tau and required more than one kinase to be accomplished in vitro (22). In the present study, we used an adenovirus-mediated gene expression system to synergistically express tau, ΔMEKK, JNK3, and GSK-3β. This system enabled us to produce hyperphosphorylation of tau in cultured cells including that AT100 and PS422. This hyperphosphorylated tau formed aggregates that were detergent-insoluble and Thioflavin-S-reactive and displayed relatively shorter fibrils than PHF. Our results suggest that the aberrant phosphorylation of tau can contribute to the formation of oligomeric tau fibrils and that other additional factors may be required for the growth step of tau fibrils.

**EXPERIMENTAL PROCEDURES**

*Generation of Recombinant Adenoviruses—All transcription units were ligated into cassette cosmID pAXCawt (TaKaRa) digested with Ssbl, so that the insert was transcribed under the control of the CAG promoter (23). Using PCR and Pyrobest DNA polymerase (TaKaRa) with pFLAG-CMV2-JNK3 (24) as a template, the mouse JNK3 open reading frame was amplified along with the FLAG tag primer (5′-ACCATGGACTACAAAGACGATGACGAC-3′) and the arm primer of

42060 This paper is available on line at http://www.jbc.org
activated JNK3 can phosphorylate tau in COS-7 cells—Because JNK is activated through ΔMEKK (35–38), we first investigated the ability of JNK3 to phosphorylate tau in the presence or absence of ΔMEKK (Fig. 1). Four different combinations of cDNAs (longest human tau with JNK3 and/or ΔMEKK or with LacZ) were introduced into COS-7 cells by using the adenovirus-mediated gene transfer method (Fig. 1).
All of the cells expressed significant levels of tau, JNK3, ΔMEKK, and/or LacZ (100% expression efficiency was obtained when the MOI of each construct used was over 10; data not shown). Cells co-expressing tau and JNK3 exhibited only negligible levels of activated forms of JNK (Fig. 1B, lane 1). In contrast, cells co-expressing tau, JNK3, and ΔMEKK exhibited significant levels of activated JNK (Fig. 1B, lane 2). JNK activation by ΔMEKK was associated with the reduced mobility of tau in SDS-PAGE and the reduced Tau-1-immunoreactivity (Fig. 1, C and D, lane 2) when compared with those in cells co-expressing tau with JNK3 alone, ΔMEKK alone, or LacZ (Fig. 1, lanes 1, 3, and 4). These results suggest that ΔMEKK activation is required for JNK3 to phosphorylate tau in COS-7 cells.

Determination of Tau Phosphorylation Sites in COS-7 Cells Expressing Activated JNK3—We further analyzed phosphorylated tau epitopes in cells co-expressing tau, JNK3, and ΔMEKK using the well characterized phosphorylation dependent anti-tau antibodies PS199, PT205, PT231, PS262, PS396, PS404, PS422, AT8, AT100, AT180, and AT270 (Fig. 2). The level of tau phosphorylation at each site was quantified as a ratio of immunoreactivity of each antibody to that of phospho-independent tau antibody TauC (Fig. 2a). Images of Western blots are shown in the lower panels of Fig. 2, and the graphs showing the corresponding quantification are shown in the upper panels. The levels of phosphorylation at PT205, PT231, PS396, AT8, AT180, AT270, PS422, and AT100 sites were significantly increased in cells expressing activated JNK3 (Fig. 2, b–i). In particular, tau phosphorylation at PT205, AT8, and PS422 was dramatically increased in response to JNK3 activation. Activation of JNK3 by ΔMEKK (MOI = 100) resulted in a 77-fold increase in PT205 immunoreactivity, an 81-fold increase in AT8 immunoreactivity, and a 330-fold increase in PS422 immunoreactivity (Fig. 2, b, e, and h, lane 3, respectively) when compared with those from cells co-infected with LacZ (MOI = 100). AT100 immunoreactivity in these cells displayed an 18-fold increase over that in control cells expressing LacZ (Fig. 2i, lane 3). Interestingly, AT100 as well as PS422 epitopes are known to be more specific in Alzheimer tau (21, 22). The immunoreactivities of PT231, PS396, AT180, and AT270 in activated JNK3-expressing cells (Fig. 2, c, d, f, and g, lane 3, respectively) exhibited 3–5-fold increases over those in control cells. PS199, PS262, and PS404 immunoreactivities increased only 1–1.8-fold, suggesting that these epitopes are not major phosphorylation sites for JNK3 (Fig. 2, j–l, lane 3, respectively). Thus, with JNK3 activation, the Ser202, Thr205, and Ser214 sites were most susceptible, the Thr231 and Ser214 sites were moderately susceptible, and the Thr212, Thr231, Ser235, and Ser265 sites were relatively less but significantly susceptible to JNK3-mediated phosphorylation. The Ser199, Ser202, and Ser404 sites appear to be unsusceptible to JNK3 phosphorylation.

HPF-like Aberrant Tau Phosphorylation by GSK-3β and Activated JNK3—JNK3 phosphorylates tau at most of the pathological sites except Ser199, Ser262, and Ser404. These JNK3-sensitive or -insensitive sites overlapped with those reported in a previous in vitro study using recombinant proteins (39). In the same study, GSK-3β was reported to phosphorylate some of the JNK-insensitive sites. Motivated by this finding, we next attempted to simultaneously express tau, ΔMEKK, JNK3, and GSK-3β in COS-7 cells to determine the effect of simultaneous overexpression of activated JNK3 and GSK-3β on tau phosphorylation in our model. We refer to this co-expression as quadruple expression in this paper.

Fig. 3a shows a result from the Western blot analysis probed with the phospho-independent anti-tau antibody TauC. With quadruple expression, tau migrated more slowly almost as a single band than tau phosphorylated solely by activated JNK3 (Fig. 3a, lanes 1 and 2), suggesting that additional overexpression of GSK-3β to activated JNK3 causes additive effect on tau phosphorylation in COS-7 cells. The Western blot analysis using the PS199, PS262, and PS404 antibodies indicated the enhanced phosphorylation at these JNK3-insensitive sites (Fig. 3, b–d). Moreover, other sites also displayed enhanced phosphorylation in response to additional GSK-3β expression. For example, the immunoreactivities of AT8 (Fig. 3g), AT180 (Fig. 3e), and AT100 (Fig. 3f) with quadruple expression increased 2–3.4-fold compared with those with activated JNK3 expression alone. In contrast, additional GSK-3β expression had no significant additive effect on the AT270 (Fig. 3f) and PS422 (Fig. 3h) immunoreactivities. Taken together, activated JNK3 and GSK-3β synergistically phosphorylated tau at most of the pathological phosphorylation sites of tau in COS-7 cells.

Histological and Biochemical Analysis of Tau in COS-7 Cells Expressing Tau, ΔMEKK, JNK3, and GSK-3β—To determine whether this quadruple expression in COS-7 cells induces aberrant tau aggregates, cells were stained with the phospho-dependent anti-tau antibody AT8 (Fig. 4b) and Thioflavin-S histochemistry (Fig. 4a). Thioflavin-S is known as a marker for the insoluble protein aggregates with β-sheet structures, as is shown in Fig. 4g, displaying an NFT-bearing neuron in an AD brain. AT8 staining was confined mainly to the cytoplasm, probably showing a partial aggregation of quadruple expression, cells were stained with the phospho-independent anti-tau antibody TauC. Al-
though the total amounts of tau recovered in the RIPA-soluble fractions were almost constant, mobility of tau in each lane changed depending on the combination of kinases expressed.

RIPA-insoluble materials were next solubilized using 70% formic acid and dissolved into Laemmli sample buffer after lyophilization. Only tau from quadruple-expressing cells was recovered in the formic acid fraction (Fig. 5b), suggesting that tau from quadruple-expressing cells becomes partly insoluble in RIPA (containing 1% SDS).

The ultrastructure of immunolabeled RIPA insoluble materials from quadruple expressing cells and AD brains were investigated by using the electron microscope. We found RIPA-insoluble tau filaments from AD brains identified with the AT8 and AT100 antibodies (Fig. 5c and e, respectively) that had two different shapes with different diameters: short fibrils with 10-nm diameter (Fig. 5c) and relatively longer fibrils with 20-nm diameter (Fig. 5e). Consistent with this observation, in the RIPA-insoluble fractions of quadruple-expressing cells, the AT8- and AT100-positive tau aggregates were identified that contain short fibril-like structures with about 10-nm diameters (Fig. 5d and f, respectively). The similar tau aggregates were found in the perinuclear regions of the quadruple expressing cells (Fig. 5g) but not in control cells expressing tau and LacZ (Fig. 5h). These data suggest that hyperphosphorylation of tau by GSK-3β and activated JNK3 could enforce the formation of tau aggregates sometimes containing short fibrils.
DISCUSSION

In this report, we demonstrate that activated JNK3 phosphorylates tau in COS-7 cells. This kinase increases levels of tau phosphorylation at the epitopes of PT205, PT231, PS396, PS422, AT8, AT100, AT180, and AT270. This is almost consistent with the results obtained from the in vitro studies (40). The combination of activated JNK3 and GSK-3β cooperatively phosphorylates 12 Ser and Thr residues of tau in vivo. These include the AT100 site, which is the specific phosphorylation site in PHF tau. There are more than 19 phosphorylation sites identified in PHF tau (41). Most phosphorylation-dependent tau antibodies, however, also have been known to recognize a fraction of tau from biopsied normal adult or fetal brains, although the levels of phosphorylation are less than those recognized in AD brains (42). Among these antibodies, AT100 never recognizes normal adult or fetal tau but does specifically recognize PHF tau (22, 42). Thus, the AT100 epitope is a unique and specific phosphorylation site in PHF tau. In this sense, tau in quadruple expressing cells is in a similar phosphorylation state to that in AD.

The in vitro phosphorylation of AT100 epitope was reported by using the combination of protein kinase A and GSK-3β (22). According to this study, the phosphorylation of the AT100 epitope required the sequential phosphorylation of GSK-3β and protein kinase A, although the other combination may be due to the phosphorylation of these epitopes. This combination may not be relevant to AD tau formation, because neither GSK-3β nor protein kinase A can phosphorylate Ser422, a site that is highly phosphorylated in AD brain (20, 21). In our study, activated JNK3 phosphorylated tau both at the Ser422 and AT100 epitopes. However, activated JNK3 did not phosphorylate the Ser199, Ser262, and Ser404 sites, phosphorylation of which required the additional expression of GSK-3β. Furthermore, immunoreactivity for AT100 epitopes is much more enhanced in cells co-expressing both activated JNK3 and GSK-3β. It is also possible that the formation of AT100 epitopes in cells expressing only activated JNK3 may result from the synergism between JNK3 and endogenous GSK-3β. Thus, the combination of GSK-3β and activated JNK3 can phosphorylate tau at most of the phosphorylation sites documented in AD. Recent studies reported that activated phospho-JNK or GSK-3β co-localized in NFT-bearing (18, 19) or AT8-positive neurons (19, 43, 44), and amyloid β treatment activated JNK (45, 46) and GSK-3β in cultured cells (47–50). Taken together, activated JNK and GSK-3β may be a strong candidate kinase combination involved in the mechanisms of tau pathology in AD.

It has been a question for a long time whether the aberrant phosphorylation of tau is a cause of NFT formation or just a consequence of some other unidentified process. One hint was
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provided by a recent in vitro study that showed that hyperphosphorylation of tau in AD brain induces self-assembly of tau into PHF (51). Our present results indicate that the aberrant phosphorylation of tau results in the formation of short fibrils that are RIPA-insoluble. These fibrils were relatively shorter than reported PHF in AD brains. Since similar types of fibrils were also recovered from AD brains, tau fibrils observed in the quadruple expressing COS-7 cells might be a good model to study tau aggregation in a cellular environment.

The formation of NFTs is mainly divided into three stages. First is the pretangle stage, which exhibits PHF epitopes and becomes Gallyas silver-positive. Second is the mature tangle stage, which shows PHF epitopes, Gallyas silver staining, and AT8 and Thioflavin-S-positive staining; Thioflavin-S becomes Gallyas silver-positive. Third is the mature tangle stage, which shows PHF epitopes, Gallyas silver staining, and AT8 and Thioflavin-S-positive staining; Thioflavin-S becomes Gallyas silver-positive.

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