Tau impacts on growth-factor-stimulated actin remodeling

Vandana M. Sharma, Joel M. Litersky, Kiran Bhaskar* and Gloria Lee‡

Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA
*Present address: Department of Neurosciences, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
‡Author for correspondence (e-mail: gloria-lee@uiowa.edu)

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Summary

The microtubule-associated protein tau interacts with the SH3 domain of non-receptor Src family protein tyrosine kinases. A potential consequence of the SH3 interaction is the upregulation of tyrosine kinase activity. Here we investigated the activation of Src or Fyn by tau, both in vitro and in vivo. Tau increased the kinase activity in in vitro assays and in transfected COS7 cells. In platelet-derived growth factor (PDGF)-stimulated fibroblasts, tau appeared to prime Src for activation following PDGF stimulation, as reflected by changes in Src-mediated actin rearrangements. In addition, while fibroblasts normally recovered actin stress fibers by 5-7 hours after PDGF stimulation, tau-expressing cells showed sustained actin breakdown. Microtubule association by tau was not required for the observed changes in actin morphology. Inhibition of Src kinases or a mutant deficient in Src interaction reduced the effects, implicating Src family protein tyrosine kinases as a mediator of the effects of tau on actin rearrangements. Our results provide evidence that the interaction of tau with Src upregulates tyrosine kinase activity and that this interaction allows tau to impact on growth-factor-induced actin remodeling.

Key words: Tau, Src, PDGF, Actin stress fibers

Introduction

Tau, MAP2, and MAP4 are microtubule-associated proteins that share a similar domain structure. All contain 3-4 copies of a microtubule-binding motif in their C-terminal domain and a proline rich domain upstream of the microtubule binding region. Although tau and MAP2 have been widely known for their role in microtubule assembly in neurons, other functions have been identified suggesting that they may be multifunctional proteins. For instance, tau can activate PLC-γ in vitro (Hwang et al., 1996) and bind to Pin1 (Lu et al., 1999). We have previously reported that tau interacts with the SH3 domains of Src family tyrosine kinases (SFKs) and that complexes of tau and Fyn are present in cells, suggesting that tau participates in signal transduction pathways (Lee et al., 1998).

In many cell types, SFKs participate in a wide array of signaling pathways (reviewed by Thomas and Brugge, 1997). Src and Fyn are critical for neuronal development as mice deficient in both Src and Fyn had defects in axonal guidance (Morse et al., 1998) and inhibition of SFKs affected growth cone steering (Suter and Forscher, 2001). Axonogenesis and growth cone motility were also reduced by dominant-negative Src (Robles et al., 2005). Similarly, the depletion of tau hindered axonogenesis (Caceres and Kosik, 1990; Dawson et al., 2001). Therefore, our discovery of an interaction between tau and SFKs raises the possibility that this interaction might have importance in axonal growth. In addition, since tau is also present in non-neuronal cells (Gu et al., 1996), the tau-SFK interaction represents a potential function for non-neuronal tau that would be distinct from neurite outgrowth.

SFKs possess SH3 and SH2 domains that engage in protein-protein interactions, with the SH3 domain recognizing proline-rich motifs and the SH2 domain recognizing phospho-tyrosine motifs. At the same time, these domains are involved in intramolecular interactions that regulate SFK activity. In the inactive conformation, the SH3 domain interacts with sequences between the SH2 and kinase domains (Sicheri et al., 1997; Xu et al., 1997) and kinase activation involves the disassembly of these intra-molecular associations and the phosphorylation of Tyr416, which is located in the activation loop of the kinase domain. Thus, the SH3 domain has a role in the regulation of Src catalytic activity (reviewed by Sicheri and Kuriyan, 1997). Moreover, the displacement of the intramolecular interactions of the SH3 domain by interactions with high affinity ligands can affect kinase activity. The upregulation of SFK activity through the displacement of the SH3 domain by ligand binding was initially described for the SH3 ligands Sin and Nef, which bind to Src and Hck, respectively (Alexandropoulos and Baltimore, 1996; Moarefi et al., 1997). Subsequently, other SH3 ligands such as β-arrestin (Luttrell et al., 1999), synapsin I (Onofri et al., 1997), and the metalloprotease-disintegrin ADAM 12 (Kang et al., 2000) have been reported to increase Src activity.

In the course of investigating the interaction between tau and SFKs, we discovered a similar function for tau. In this report we show that tau enhances the kinase activity of Fyn and Src in vitro and in co-transfected cells. We then used PDGF signaling in 3T3 cells as a means to demonstrate an effect of tau on endogenous Src activity. We found that tau expression altered PDGF-induced cytoskeletal rearrangements and that this effect appeared to be mediated, in part, by Src. Our data suggest that the association between tau and Src might serve as a mechanism for coupling extracellular signals to the cytoskeletal system.

Results

Tau affects SFK activity

We have previously shown through in vitro binding assays that human tau interacted with the SH3 domain of Fyn and Src (Bhaskar et al., 2005; Lee et al., 1998). Based on the crystal structure of Src, SH3 ligands are thought to 'unclamp' an inactive form of Src, enhancing its activation (Xu et al., 1999; Xu et al., 1997). Our first indication that tau might function in this manner came from experiments initially performed to examine the ability of tyrosine-phosphorylated tau to associate with microtubules. Purified tau and Fyn were incubated in an in vitro kinase reaction to first generate tyrosine-phosphorylated tau. The reaction was then added to taxol-stabilized microtubules. Upon examining the microtubule pellet for tyrosine-phosphorylated tau, we discovered that the taxol-stabilized tubulin was also tyrosine phosphorylated, presumably by the Fyn that had been carried over from the kinase reaction. Moreover, although Fyn alone was capable of phosphorylating taxol-stabilized microtubules, the presence of tau greatly enhanced the phosphorylation (Fig. 1A, compare lanes 4 and 8).

However, because tau binds to microtubules, a possible explanation for the enhanced tubulin phosphorylation was that tau was simply bringing Fyn to the microtubules and facilitating tubulin phosphorylation without affecting Fyn activity. This would predict that a fragment of tau lacking microtubule-binding activity would be incapable of enhancing the phosphorylation. We therefore tested an N-terminal fragment of tau that binds to Fyn SH3 but lacks the microtubule-binding repeats [amino acids 1-184 (Lee et al., 1998)]. By using the same protocol in which the tau fragment was incubated with Fyn and then added to taxol-stabilized microtubules, we found that the N-terminal fragment was similarly capable of enhancing the phosphorylation of tubulin (Fig. 1B). This suggested that the increase in phosphorylation was mediated by an interaction between Fyn and the N-terminus of tau; microtubule binding by tau was not required.

Next, we sought to determine the effect of tau on Fyn activity in a transfected cell system. We co-transfected tau and/or Fyn into COS7 cells, purified the microtubules by taxol driven assembly, and then probed the microtubules for tyrosine phosphorylation. While the expression of Fyn alone was sufficient to phosphorylate tubulin, the presence of tau increased the level of phosphorylation (Fig. 1C, top). The increase was ~50%, as averaged from three experiments. The tubulin probe confirmed that tubulin levels in the Fyn alone and the fyn plus tau transfections were similar (Fig. 1C bottom). Taken together, these results indicate that tau can enhance Fyn kinase activity for tubulin both in vitro and in cells.

As tau also interacts with the SH3 domain of Src (Lee et al., 1998), we tested tau for its ability to similarly enhance tubulin phosphorylation by Src in vitro. Tyrosine phosphorylation of tubulin by Src was increased when the Src had been pre-incubated with tau (Fig. 1D, compare lanes 2 and 4). As an indication of tau's ability to increase Src activity, we also investigated the effect of tau on the autophosphorylation of Src at Tyr416, which has been proposed to take place following the disruption of intramolecular SH3/SH2 interactions (Xu et al., 1999). Src kinase was incubated with and without tau and then probed for the autophosphorylation of Tyr416 with a phospho-specific antibody. Without tau, phospho-Tyr416 immunoreactivity was absent, which suggested that the Src preparation, by itself, was in an inactive state and could not autophosphorylate Tyr416 (Fig. 1E, lane 3). As tau was added, we observed the autophosphorylation of Src at Tyr416, with an increase in phospho-Tyr416 observed when tau was increased (Fig. 1E, lanes 1, 2). These results are consistent with the hypothesis that, in Src, conformational changes induced by SH3 ligand binding precede the autophosphorylation of Tyr416.

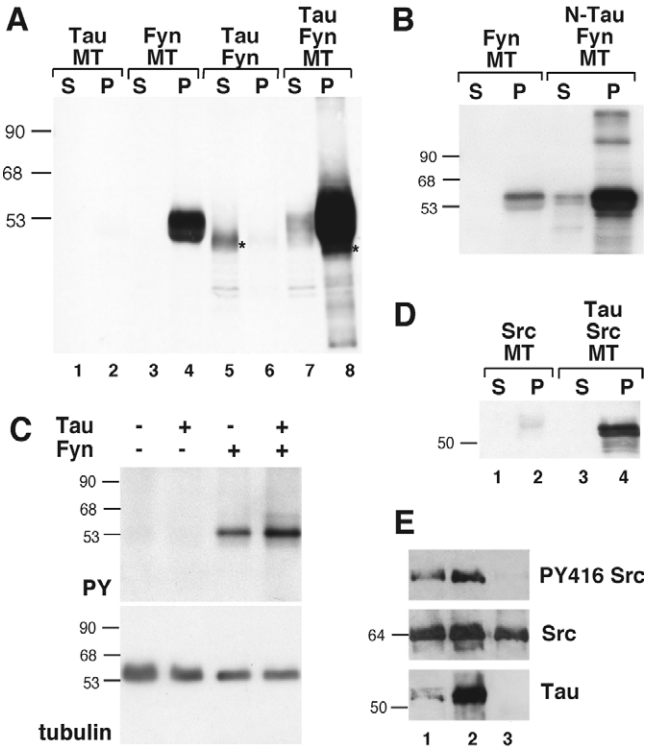

Fig. 1. Tau increases SFK activity. (A-D) Tyrosine phosphorylation of tubulin in the presence of tau and Fyn or Src. (A) *E. coli* tau and/or Fyn were incubated and added to microtubules. After centrifugation, the supernatant (S) and microtubule pellet (P) were probed with anti-phosphotyrosine (see Materials and Methods). While microtubules were phosphorylated by Fyn (lane 4), tau increased the level of phosphorylation (lane 8). Autorad was deliberately overexposed to show tyrosine phosphorylated tau in lanes 5 and 8 (asterisk). (B) N-terminal fragment of tau and Fyn were incubated, added to microtubules, and probed as described in A. (C) Microtubules isolated from COS7 cells transfected with full-length tau and/or Fyn were probed with anti-phosphotyrosine (top panel) or anti-tubulin (bottom panel). (D) Tau and Src were incubated, added to microtubules, and probed as described in A. (E) Tau activates autophosphorylation of Src. 0.2 or 2 μM tau (lanes 1 and 2, respectively) was incubated with Src and probed for active Src, total Src, and tau as described in Materials and Methods.

Tau affects PDGF-induced alterations in actin morphology

To investigate the ability of tau to affect endogenous Src activity in a cellular system, we turned to PDGF-stimulated 3T3 cells. The response of 3T3 cells to PDGF has been extensively investigated, with Src known to play a central role in the PDGF-activated signal transduction pathway in these

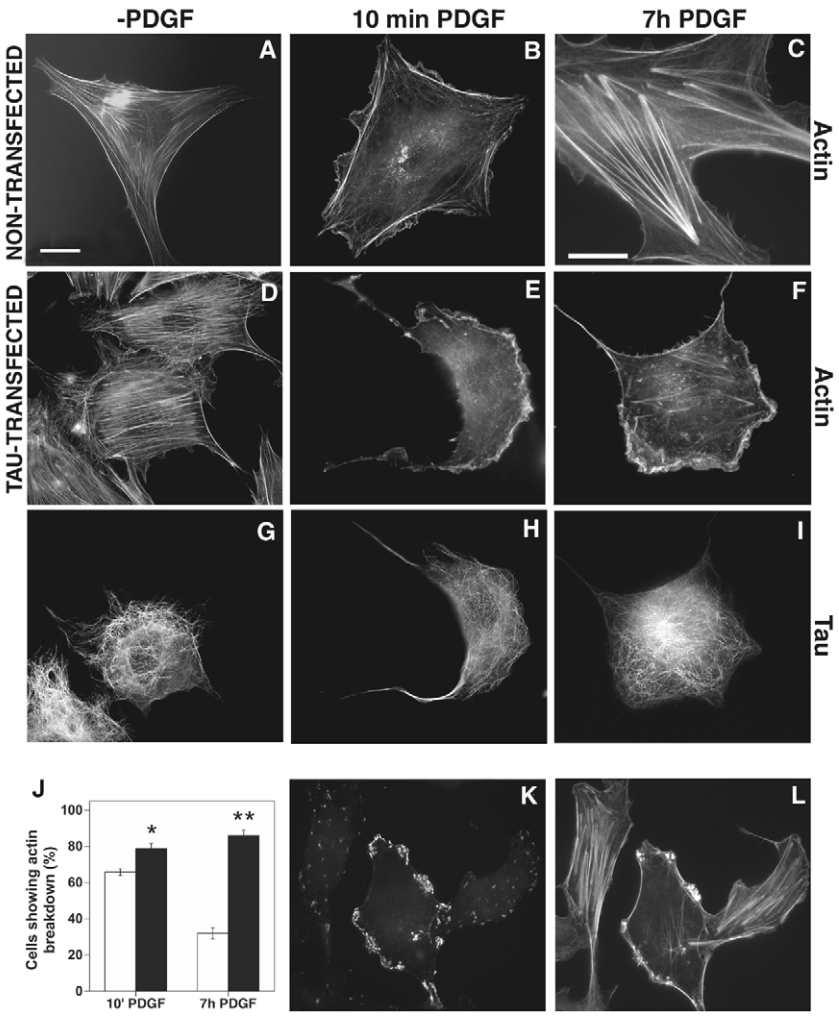

Fig. 2. Tau expression causes changes in actin reorganization following PDGF stimulation. 3T3 cells transiently transfected with tau were stimulated with PDGF for 10 minutes or 7 hours, fixed and labeled with phalloidin-Alexa594. Panels A-C show the actin morphology of non-transfected control cells following PDGF treatment as indicated. Panels D-F show the actin morphology in tau-expressing cells similarly treated. Tau-transfected cells were identified by labeling with the polyclonal tau antibody CR (G-I). (J) Quantification of cells showing PDGF-induced actin fiber breakdown in the presence and absence of tau. Percentage of non-transfected cells (open bars) and tau-transfected cells (black bars) showing actin stress fiber breakdown was determined after either 10 minutes or 7 hours PDGF treatment as described in Materials and Methods. (K-L) Expression of a constitutively active form of Src also disorganized the actin cytoskeleton. Labeling for activated Src (phospho-Y416) (K) and phalloidin (L) are shown. Bar in A, 10 μm (applies to panels A, B, D-I). Bar in C, 10 μm.

cells (reviewed by Abram and Courtneidge, 2000; Parsons and Parsons, 1997). In this widely used system, within minutes after PDGF addition, a dramatic reorganization of the cytoskeleton takes place with the loss of stress fibers and the formation of membrane ruffles (Ridley and Hall, 1992; Ridley et al., 1992). Several hours after PDGF addition, the cell recovers and regains stress fibers.

To test the effects of tau on Src activity in cells, we transiently expressed tau in 3T3 cells, which lack endogenous tau, and then stimulated the cells with PDGF. At various times after stimulation, cells were fixed and the actin stress fibers were examined. As expected, after 10 minutes of PDGF stimulation, non-transfected 3T3 cells exhibited membrane ruffling and the loss of actin stress fibers; tau-expressing cells responded in a similar manner (Fig. 2B,E). Because of the heterogeneity in cell response, we quantified the proportion of cells exhibiting actin stress fiber breakdown. Cells that lacked the normal stress fiber pattern were scored positive for loss of stress fibers; these cells had either a few isolated stress fibers, as in Fig. 2B or 2F, or no stress fibers as in Fig. 2E. We found that 65±2% of non-transfected cells and 79±3% of tau transfected cells showed the loss of stress fibers 10 minutes after PDGF treatment. The small increase in the proportion of tau-transfected cells with actin breakdown was statistically significant (P<0.05). However, after 5-7 hours of PDGF treatment, a more striking difference emerged. Although most of the non-transfected cells had re-formed their stress fibers (Fig. 2C), the presence of tau caused a sustained loss of stress fibers and persistent ruffling (Fig. 2F). Quantification showed that while actin stress fibers were restored in 68% of the non-transfected cells, only 14% of tau-expressing cells had recovered stress fibers (Fig. 2J shows the corresponding percentages of cells showing the loss of actin stress fibers – 32±3% of the non-transfected cells and 86±3% of the transfected cells at 7 hours PDGF). The morphology of the cells with the loss of stress fibers was similar to that obtained when a constitutively active form of Src kinase was expressed in 3T3 cells [Fig. 2L (Felice et al., 1990)]. As a control, we also noted that, in the absence of PDGF, the expression of tau did not cause changes in actin organization (Fig. 2D).

Tau-linked alterations in the actin cytoskeleton are independent of its microtubule-stabilizing effect

Microtubule dynamics have been reported to affect actin rearrangements (Buck and Zheng, 2002) and since tau is known to stabilize microtubules, the observed effects of tau could occur as a consequence of its ability to stabilize microtubules. To address this possibility, 3T3 cells were treated with a low concentration of taxol to stabilize microtubules prior to PDGF stimulation. [Our previous studies had shown that 0.1

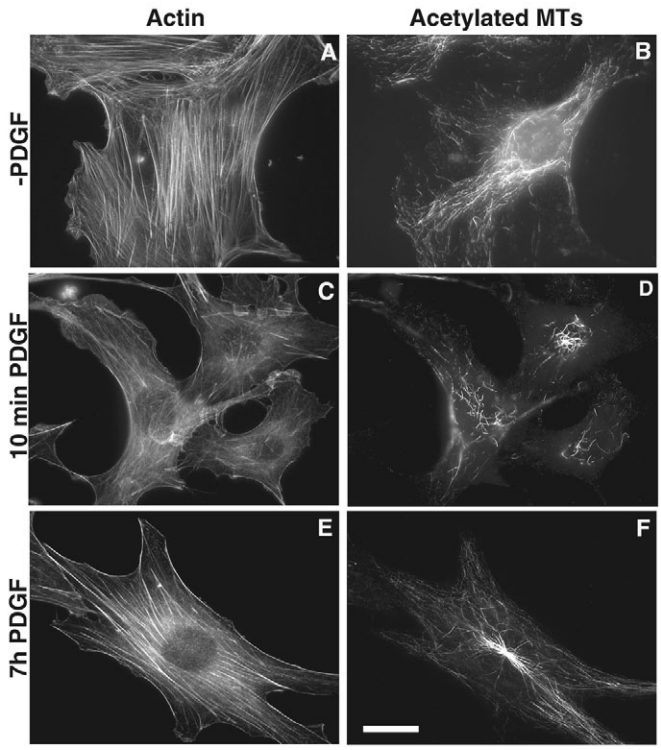

Fig. 3. Taxol treatment of 3T3 cells does not affect PDGF-induced actin reorganization. 3T3 cells treated with 100 nM taxol were stimulated with PDGF and double-labeled with Phalloidin-Alexa594 (A,C,E) and anti-acetylated tubulin (B,D,F). Bar, 10 μm.

μM taxol treatment, rather than the conventionally used 10 μM, was most effective in duplicating the effects of tau transfection (Leger et al., 1994).] First, to confirm that our taxol conditions were capable of stabilizing microtubules, we examined the acetylation of microtubules in this system. As expected from the known effects of taxol, the acetylated microtubule array was extensive (Fig. 3B), unlike the more restricted patterns of curly microtubules seen in normal 3T3 cells (Piperno et al., 1987). However, PDGF treatment broke down the acetylated tubulin array (Fig. 3D), supporting the notion that, while the 0.1 μM taxol condition increases microtubule stabilization, the ability of microtubules to grow and shrink is retained (Derry et al., 1995). In addition, we found that 0.1 μM taxol-treated cells responded to PDGF in the same manner as untreated 3T3 cells; stress fibers were lost after 10 minutes of PDGF stimulation and recovered after 7 hours (Fig. 3A,C,E). Therefore, the tau effect on PDGF-stimulated 3T3 cells was distinct from that of the 0.1 μM taxol condition, suggesting that the effect of tau on actin rearrangement was distinct from a microtubule-stabilizing effect.

As a separate approach, we tested tau that lacked microtubule-binding activity by using an S262D/S356D mutant of tau. This mutant mimics the phosphorylation of tau by MARK or Par1 kinase, which has been shown to inhibit the association of tau to microtubules (Biernat et al., 1993). As expected, the mutant tau showed a diffuse localization in the cytosol, with little co-localization to microtubules (Fig. 4D-F). After PDGF stimulation, we found that actin rearrangements induced in cells expressing the mutant tau were similar to those seen with wild-type tau, with loss of stress fibers and ruffling visible at the 7 hour time point (Fig. 4A-C). 79% of the transfected cells failed to recover stress fibers after 7 hours of PDGF stimulation, supporting the notion that the observed effects of tau on actin morphology are independent of its microtubule binding ability.

Tau-linked alterations in the actin cytoskeleton correlate with alterations in Src localization and activity

Upon activation, Src has been shown to translocate to the cell periphery (Fincham et al., 1996). We examined the distribution of activated Src in 3T3 cells after PDGF stimulation using an antibody that recognizes Src phosphorylated on Tyr416. In non-stimulated cells, activated Src was localized to focal adhesions (Fig. 5A); Src associates with the termini of the actin stress fibers in these structures (Fincham and Frame, 1998). After 10 minutes of PDGF stimulation, activated Src translocated to the cell periphery and was distributed in a punctate pattern along the cell edge (Fig. 5B). After 7 hours of PDGF stimulation, the activated Src distribution in non-transfected cells reverted to the quiescent pattern (Fig. 5C). By contrast, double-labeling with anti-tau and anti-Src-phospho-Tyr416 demonstrated that, in tau-transfected cells, much activated Src was still found at the cell edge after 7 hours of PDGF stimulation (Fig. 5F). At this time point, we also noted that the absence of the focal adhesion pattern for activated Src coincided with the absence of actin stress fiber recovery in the

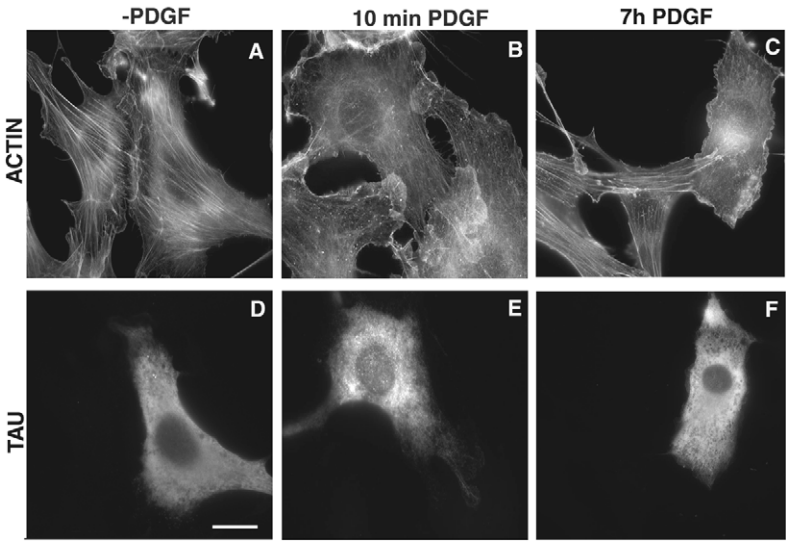

Fig. 4. Loss of microtubule-binding activity does not alter the effects of tau on PDGF-induced actin reorganization. 3T3 cells were transfected with S262D/S356D mutant tau, stimulated with PDGF, and double-labeled as described in Fig. 2 (phalloidin-Alexa594 in A,B,C and anti-tau in D,E,F). The mutated tau exhibited a diffuse cytoplasmic distribution due to its loss of microtubule binding. Bar, 10 μm.

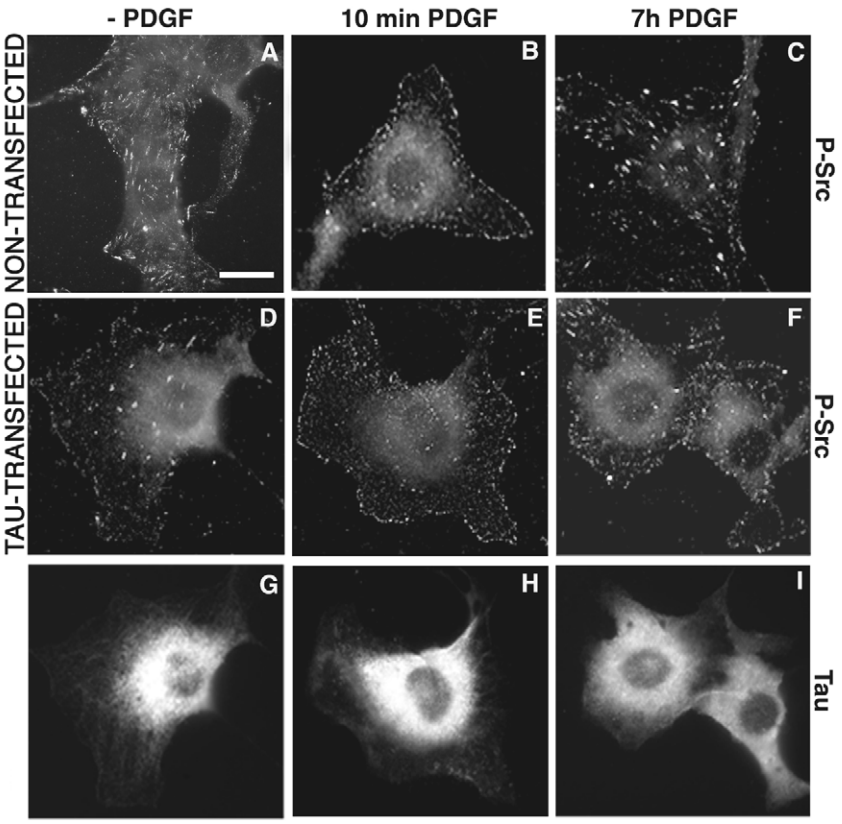

Fig. 5. Activated Src distribution in tau-transfected 3T3 cells following PDGF stimulation. 3T3 cells were transfected with tau, stimulated with PDGF, and double-labeled with anti-Src (phospho-Y416) (A-F) and anti-tau (G-I). Quiescent cells showed activated Src located in focal adhesions (Fincham and Frame, 1998) (A,D). Upon stimulation with PDGF, activated Src localized to the periphery in a punctate pattern (B,E). At 7 hours, while the Src distribution reverted to the focal adhesions in non-transfected cells (C), in tau-expressing cells, much activated Src was still localized at the periphery (F). The tau distribution (G-I) was non-fibrillar owing to the fixation conditions required for labeling with the activated Src antibody. Bar, 10 μm.

interior of the cells. That is, the sustained localization of activated Src at the cell periphery correlated with the loss of actin stress fibers.

As an aside, it should be noted that the antibody recognizing activated Src will also recognize activated Fyn and Yes. However, the localization of activated Fyn and Yes in 3T3 cells has not been described whereas the staining patterns that we had obtained with anti-Src-phospho-Tyr416 were consistent with the known localizations for activated Src in PDGF-treated 3T3 cells. Therefore, we interpreted the anti-Src-phospho-Tyr416 staining to indicate the presence of activated Src, at the very least. Our results at 7 hours after PDGF stimulation suggest that the presence of tau alters Src activity.

Src activity is known to be involved in the breakdown of focal adhesions and the reorganization of the actin cytoskeleton at the cell periphery (reviewed by Frame et al., 2002) and in 3T3 cells, Src has been shown to be the SFK that is most critically involved in PDGF-stimulated actin rearrangements and chemotaxis (Shah and Vincent, 2005). To investigate the involvement of Src in mediating the effects of tau on actin rearrangements, we treated the cells with PP2, a pharmacological inhibitor of SFKs. The SFK inhibitor PP2 binds at the ATP-binding site of SFK and effectively locks the catalytic site in a conformation that does not allow the activation loop to become phosphorylated at Tyr416 (Schindler et al., 1999). Therefore, the presence of tau would not be expected to prevent the action of PP2. In our experiments, PP2 was added at different time points relative to PDGF treatment followed by actin visualization; the different protocols of treatment are shown schematically in Fig. 6 (top panel). Using the first protocol (Protocol 1), we found that the addition of 10 μM PP2 simultaneously with PDGF for 10 minutes impaired the ability of both tau-transfected and non-transfected cells to break down actin stress fibers and to form membrane ruffles in response to PDGF stimulation (compare Fig. 6B with Fig. 2B,E). Non-transfected and transfected cells were similarly inhibited, with 24±6% and 29±7%, respectively, showing actin stress fiber loss. Both numbers represented a reduction of around 60% in the proportion of responding cells when compared to cells without PP2 treatment (compare Fig. 6G 'PP2+PDGF' with '10' PDGF'). Anti-Src-phospho-Tyr416 staining was barely detectable, confirming the inhibition of Src activity by PP2 (not shown). These data confirmed that the presence of tau did not neutralize the effects of PP2.

As a second protocol of treatment, we pretreated 3T3 cells with PP2 for 45 minutes prior to PDGF addition, and then examined the cells 10 minutes after PDGF stimulation (Protocol 2). Using this protocol, we found that 51±2% of non-transfected cells responded to PDGF, as indicated by stress fiber breakdown. Relative to cells without PP2 treatment, the reduction in response was 21%. Since the PP2 pretreatment resulted in a lower level of inhibition relative to that obtained when PP2 was added with PDGF, this suggested that PP2 inhibition was transitory, possibly due to cellular breakdown of PP2. By contrast, the transfected cells showed no reduction in response when PDGF was added 45 minutes after PP2 addition (Fig. 6C,D), with 84±6% transfected cells showing loss of stress fibers. These data suggest that the presence of tau opposes the effects of PP2. As PP2 inhibits SFKs, these data support the hypothesis that tau enhances or facilitates SFK activation.

Our third PP2 protocol addressed the question of whether the persistent loss of stress fibers exhibited by 86% of the tau transfected cells 5-7 hours after PDGF stimulation depended on the activity of SFKs. If so, inhibition by PP2 should allow stress fibers to re-form. Tau transfected cells were stimulated with PDGF and then after 5 hours, the cells were treated with PP2 for 30 minutes (Protocol 3). Inhibition of Src at this stage allowed stress fibers to re-form in tau-expressing cells (Fig. 6F), decreasing the percentage of cells with actin breakdown to 56%, which corresponded to a 34% reduction relative to the

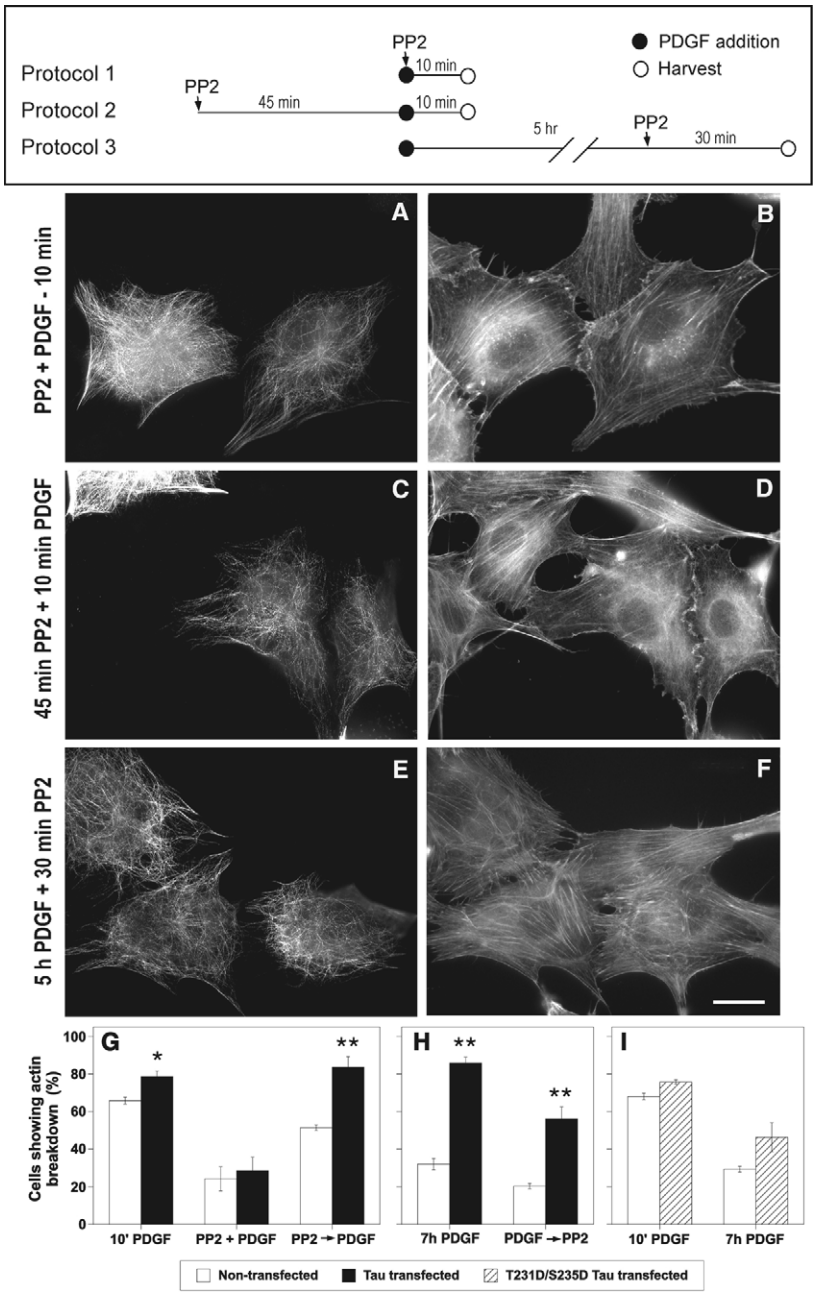

Fig. 6. Effect of Src inhibition on tau-induced alterations in actin reorganization. The top panel shows a schematic diagram of the different protocols of PP2 treatment. Micrographs show 3T3 cells transfected with tau, treated with 10 μM PP2 and PDGF, and double-labeled with anti-tau (A,C,E) and phalloidin-Alexa594 (B,D,F). Cells received either PP2 and PDGF simultaneously (A,B), PP2 45 minutes in advance of PDGF (C,D), or PP2 5 hours after PDGF addition (E,F) as described in the Results and shown in the schematic above. Bar, 10 μm. (G,H) Quantification of cells showing PDGF-induced actin fiber breakdown in the presence of PP2. Percentage of non-transfected and tau-transfected cells showing actin stress fiber breakdown in the presence of Src inhibition was determined using three protocols of PP2 treatment. PP2+PDGF indicates simultaneous addition of PP2 and PDGF with actin fiber breakdown assessed 10 minutes after addition (protocol 1). PP2→PDGF indicates 45 minutes of PP2 treatment in advance of PDGF addition, with actin fiber breakdown assessed 10 minutes after PDGF addition (protocol 2). PDGF→PP2 indicates 5 hours PDGF incubation in advance of PP2 addition, with actin fiber breakdown assessed after 30 minutes PP2 treatment (protocol 3). Data for untreated cells were reproduced from Fig. 2J for comparison. (I) Effect of the tau phosphomimicking mutant on actin stress fiber breakdown. The percentage of cells transfected with the tau T231D/S235D mutant showing actin stress fiber breakdown was determined after either 10 minutes or 7 hours PDGF treatment. Significant differences are $*P<0.05$ and $**P<0.001$, respectively, between transfected and non-transfected cell percentages. Non-transfected cells are indicated with open bars, tau-transfected cells with black (G,H) or striped (I) bars.

86%. Interestingly, a similar change was also observed in the non-transfected cells. That is, PP2 treatment at 5 hours post-PDGF also decreased the percentage of non-transfected cells exhibiting actin fiber breakdown, from 32% to 20%, which corresponded to a 36% reduction (compare Fig. 6H '7h PDGF' with 'PDGF→PP2'). The similarity in the extents of reduction for both the non-transfected and transfected cells suggested that there existed a given percentage of cells that, at any time, would contain sufficient activated Src to cause the loss of stress fibers. For example, cells at a specific stage of the cell cycle might contain more activated Src. In such cells, inhibition of Src would allow for the recovery of stress fibers. Nevertheless, there still remained 56±6% of the PP2-treated, tau-transfected cells that had not recovered actin stress fibers, as opposed to 20±1% in the non-transfected group. This suggested that there were other effects mediated by tau, unrelated to Src, that contributed to the persistent loss of stress fibers.

As an additional indication that the interaction between tau and Src contributed to the observed effects on actin morphology, we used a tau mutant that had a reduced association with Src SH3. Our previous surface plasmon resonance data had shown that phosphorylation mimicking mutations at Thr231 and Ser235 were able to reduce the association of human fetal tau with the Src SH3 domain by approximately 30 times (Bhaskar et al., 2005). Just as the T231D/S235D mutant tau protein was not phosphorylated by Src on Tyr18 (Bhaskar et al., 2005), we predicted that the T231D/S235D mutant tau would be less effective in enhancing Src activation and altering actin morphology in the 3T3 system. At both 10 minutes and 7 hours of PDGF treatment, we found

that the differences between the percentages of non-transfected and transfected cells with actin stress fiber breakdown (Fig. 6I) were not statistically significant. (The calculated *P* value for the difference at 7 hours (29.3±1.5 versus 46.3±7.8) was less than 0.06 but exceeded the 0.05 value required for statistical significance.) As the phosphomimetics reduced the effects of tau on actin morphology, these data support our hypothesis that, in this system, the interaction between tau and Src affected actin rearrangements downstream of growth factor stimulation.

Discussion

Our earlier studies have demonstrated an interaction of tau with the SH3 domain of SFKs (Lee et al., 1998). Here, we obtain evidence that a functional consequence of this interaction is the upregulation of tyrosine kinase activity. Our data support the hypothesis that SH3 ligands can uncouple intramolecular interactions within the inactive Src molecule, leading to conformational changes at the catalytic site of the enzyme (Moarefi et al., 1997). These changes would convert what had been a blocked substrate-binding site into an active site (Schindler et al., 1999; Xu et al., 1999).

In considering the structural motifs in tau involved in the SH3 association, we have identified a PXXP motif (residues 233-236) in tau that mediates the interaction with the Fyn SH3 domain (Lee et al., 1998). This region of tau is also involved in microtubule nucleation and is capable of interacting with the tubulin heterodimer (Brandt and Lee, 1993). For the interaction of tau with the Src SH3, while our earliest data revealed that the same PXXP had a role in the interaction, our surface plasmon resonance data have indicated that sequence in the microtubule repeat domain is also critical for the interaction (K.B. and G.L., unpublished). Therefore, since the same tau regions recognized by these domains are microtubule- and/or tubulin-binding areas, we predict that the association of a Fyn or Src SH3 domain with tau must compete with the tubulin interaction.

In our in vitro tests (Fig. 1A,B,D), we had pre-incubated tau and the SFK prior to adding microtubules; this allowed us to visualize a significant increase in kinase activity. In contrast, when tau and Fyn were co-transfected into COS cells, the extent of the increase in tubulin phosphorylation was less (Fig. 1C). This may be due to cellular microtubules being the preferred binding partner for tau, resulting in less interaction between tau and Fyn. This is consistent with our previous observations in co-transfected 3T3 cells, where the majority of tau and Fyn did not co-localize, and tau associated with microtubules and Fyn associated with cell membranes (Lee et al., 1998). Co-localization of tau and Fyn occurred primarily in highly expressing cells, where the co-localization was observed in vesicles in the cell cortex (Lee et al., 1998). In vivo, the phosphorylation of tau could also affect its association with Fyn since the tau-Fyn SH3 interaction was modulated by phosphorylation (Bhaskar et al., 2005; Zamora-Leon et al., 2001). Therefore, tau phosphorylation could regulate its ability to affect SFK activity by affecting its microtubule association and/or by affecting its SH3 association. At the same time, the integrity of microtubules would also affect the amount of tau available to affect SFK activity. Interestingly, it has been reported that colchicine-mediated microtubule disassembly caused Src activation in monocytes (Schmid-Alliana et al., 1998) and the idea that microtubules can sequester and release signaling molecules has been put forward (reviewed by Gundersen and Cook, 1999).

Further support for the ability of tau to influence SFK activity was obtained by examining the response of 3T3 cells to signaling by PDGF. In 3T3 cells, while PDGF stimulation activates a signaling cascade that includes Src, Fyn and Yes (reviewed by Heldin et al., 1998; Parsons and Parsons, 1997), Src is the SFK that is involved in PDGF-stimulated actin rearrangements (Shah and Vincent, 2005). Therefore, in our system, we viewed actin stress fiber breakdown as an indication of Src activation.

In 3T3 cells, the activation of endogenous Src is tightly regulated. Overexpression of tau did not produce any significant effects in non-stimulated cells. However, our results suggested that the presence of tau appeared to 'prime' Src for activation as a small but significant increase in the percentage of cells with actin stress fiber breakdown was found in tau-expressing cells upon PDGF stimulation. That is, the tau-SH3 interaction would induce Src molecules to adopt a conformation that allowed for more rapid or efficient activation upon PDGF addition. An increase in the outward extension of the Src activation loop may be a part of such conformational changes.

Our tests performed with PP2 further probed the relationship between PDGF stimulation, SH3 domain ligation and the activation of Src, as judged by actin stress fiber breakdown. By adding PP2 45 minutes in advance of PDGF, we were able to demonstrate a partial inhibition of Src in non-transfected cells whereas tau-transfected cells had no Src inhibition. We surmise that, owing to intracellular breakdown, the efficacy of PP2 was reduced after 45 minutes, allowing more cells to contain higher levels of uninhibited Src. In tau-transfected cells, these uninhibited Src molecules would be primed by tau leading to higher levels of activated Src upon PDGF addition. These higher levels of activated Src would then exceed the threshold level required to activate the cascade of tyrosine phosphorylation and the reorganization of the actin cytoskeleton, in spite of some PP2 being present. In the absence of tau, Src is less efficiently activated and fewer cells would produce enough activated Src to exceed the threshold level, resulting in the partial inhibition observed in our assay. When PP2 and PDGF were added together, the potency of the PP2 prevented the amount of activated Src to exceed the threshold level, despite the presence of tau.

The ability of tau to induce more actin breakdown in the presence of Src inhibition, its ability to activate SFK activity in vitro, and our results obtained with the T231D/S235D tau mutant support the hypothesis that tau can act through Src to affect actin organization. We speculate that the mechanism underlying the lack of stress fiber recovery at the 7 hour time point involves the sustained activation of Src. It is well established that Src activation leads to the inhibition of RhoA, resulting in stress fiber breakdown (Arthur et al., 2000; Timpson et al., 2001). This pathway is mediated by Src activation of p190RhoGAP, a GTPase-activating protein that inactivates RhoA (Arthur and Burridge, 2001; Brouns et al., 2001; Fincham et al., 1999). Src activation also leads to membrane ruffling and lamellipodia formation at the cell periphery, which is mediated by Rac (Timpson et al., 2001). The presence of activated Rac in tau-transfected cells 7 hours after PDGF stimulation was suggested by the membrane

ruffling shown in Fig. 2F and Fig. 4C. We also found that the tau-transfected cells had more activated SFK located at the cell periphery when compared to the non-transfected cell (Fig. 5). The pattern of staining at the periphery resembled that of the Rac-induced focal complexes located at the leading edge of lamellipodia described by Nobes and Hall (Nobes and Hall, 1995). The localization of Src to the lamellipodia is thought to require activated Rac and the N-terminus of Src, which contains the SH3 domain (Kaplan et al., 1994; Timpson et al., 2001). In addition, it was noted that the activated SFK staining in the intracellular focal adhesions appeared less dash-like (compare Fig. 5F with 5C), suggesting that focal adhesions were less developed. As stress fibers are an integral component of focal adhesions, the loss of stress fibers in tau-transfected cells could hamper focal adhesion re-formation following PDGF stimulation. Overall, our results on Src localization and actin morphology suggest that the presence of tau caused cells to retain a morphology similar to that of the activated cell, with activated Rac and inactivated Rho. The mechanisms underlying the sustained localization of activated Src to the cell periphery and the altered development of focal adhesions in tau-transfected cells remain to be elucidated.

In the PDGF-stimulated 3T3 cells, tau-mediated effects on the actin cytoskeleton that did not involve Src were revealed by PP2 treatment. When cells were treated with PP2 5 hours after PDGF addition, we found that many tau-transfected cells (56%) still lacked stress fibers. This suggested that other tau-related events downstream of Src may affect actin reorganization. That is, tau may impact on other pathways that affect the recovery of the actin cytoskeleton following PDGF stimulation. For instance, the ability of tau to antagonize the small GTP-binding protein GEM GTPase has been reported (Oyama et al., 2004). GEM is known to negatively regulate Rhoβ kinase, which is involved in the organization of the cytoskeleton (Ward et al., 2002). Other tau interactors that may impact on the actin cytoskeleton are phospholipase Cγ (Hwang et al., 1996) and Abl (Derkinderen et al., 2005).

The idea that tau might affect the actin cytoskeleton has been previously suggested by studies where tau depletion has been correlated with alterations in the morphology of the growth cone. Tau-antisense-treated neurons had a reduced growth cone area and filipodia number (DiTella et al., 1994), whereas the chromophore-assisted, laser-mediated inactivation of tau in the growth cone caused the collapse of lamellipodia (Liu et al., 1999). Our data allow us to speculate that, in tau depleted growth cones, the observed effects may have involved a decrease in activated Src that led to a decrease in Rac activity and the ability of the cell to establish or maintain lamellipodia. In agreement, local inactivation of SFKs also resulted in growth cone collapse (Robles et al., 2005) and growth cones expressing dominant negative Rac1 had reduced lamellipodial protrusions (Woo and Gomez, 2006). Most recently, in PC12 cells, tau has been localized to lamellipodia-like structures in an NGF-dependent manner, where it associated with actin (Yu and Rasenick, 2006). In addition, the N-terminus was required for the NGF-dependent aspect of the localization. Since the N-terminus of tau associates with SFK and the cellular location of tau-SFK complexes could be shifted by cytochalasin (Lee et al., 1998), SFK may be responsible for bridging tau to actin in the PC12 cells following NGF stimulation.

In primary cultured neurons, tau has been found in the growth cone where it co-localizes with dynamic micotubules (Black et al., 1996; DiTella et al., 1994). Also, the gradient of tau content in axons did not correlate with the extent of microtubule stability (Black et al., 1996). These findings suggest that in the growth cone, tau associates with dynamic microtubules without stabilizing them. The importance of a tau with reduced microtubule stabilizing activity has been supported by the finding that the phosphorylated Ser262Ser356 form of tau was found to be required for neurite outgrowth in neuroblastomas (Biernat et al., 2002). Such tau phosphorylation may release it from microtubules, allowing it to participate in signal transduction processes. In the growth cone, the activation of SFKs in the peripheral zone requires dynamic microtubules (Suter et al., 2004). Together with our data, we speculate that the dynamic microtubules are required to deliver tau to the growth cone periphery where it activates SFKs; a depletion of tau would reduce kinase activation, alter actin morphology, and most likely affect growth cone motility. Defects in axonogenesis induced by tau depletion might be explained by the failure of growth cones to achieve the threshold level of activated Src required to orchestrate the actin re-modeling that is characteristic of the highly motile axonal growth cone. In addition, the unique collection of kinase activities that are required for axonal development, such as SAD-1/PAR-1 and glycogen synthase kinase 3β (Biernat et al., 2002; Jiang et al., 2005; Kishi et al., 2005), may be intended to insure that the phosphorylation state of tau in the axon tip would favor the interaction between tau and SFK.

Our surface plasmon resonance measurements have indicated that the phosphorylation state of tau in AD, as well as the missense mutations in tau linked to FTDP-17 neurodegenerative diseases, may increase its interaction with SFK (Bhaskar et al., 2005). Our data now suggest that this could result in increased activation of SFK in the course of neuropathogenesis. It has been reported that AD brains have an increase in tyrosine phosphorylated proteins (Shapiro et al., 1991) as well as altered localizations of Fyn and Src (Ho et al., 2005; Shirazi and Wood, 1993). An increase in SFK activity would impact on synaptic transmission and plasticity (reviewed by Kalia et al., 2004) and evidence for the role of Fyn in synaptotoxicity in AD has been obtained using mouse models (Chin et al., 2005; Chin et al., 2004). Moreover, altered actin dynamics in AD brain and in Aβ treated neurons have been reported (Grace and Busciglio, 2003; Heredia et al., 2006; Maloney et al., 2005; Zhao et al., 2006). Our results raise the possibility that mutant or hyperphosphorylated forms of tau that acquire an increased ability to interact with Fyn or Src could impact on actin dynamics. Lastly, an aberrant activation of Fyn or Src in neurons could also be linked with cell cycle activation in AD (reviewed by Lee, 2005).

Materials and Methods

In vitro phosphorylation of tubulin

Human fetal tau (1 μg, synthesized by *E. coli*) and/or Fyn or Src (4-11 units, Upstate USA, Charlottesville, VA) was incubated at 37°C according to manufacturer's conditions and added to 10 μg taxol-stabilized microtubules. After a second incubation to allow microtubule binding, the reaction was layered over a sucrose cushion and centrifuged to separate microtubules. Both supernatant and microtubule pellet were examined by western blotting for the presence of tyrosine phosphorylation using monoclonal antibody 4G10 (Upstate USA). *E. coli*-synthesized N-terminal tau (1 μg, residues 1-184 of human fetal tau) was purified as previously described [(Lee et al., 1998); residue 184 corresponds to residue 242 in the longest adult tau isoform].

In vitro Src autophosphorylation assay

A kinase reaction mixture (40 μl) containing Src (11 units, Upstate USA), 25 mM $MnCl_2$, 125 mM $MgCl_2$, 2 mM EGTA, 0.25 mM $NaVO_4$, 2 mM DTT, 0.25 mM ATP and 100 mM Tris-HCl (pH 7.2) was incubated with and without tau for 6 hours at 30°C. Bacterially synthesized tau at 0.2 or 2 μM was used. Reactions were terminated by the addition of Laemmli sample buffer and boiled. Active Src was detected by western blotting with anti-Src-phospho-Tyr416 (Cell Signaling Technology, Beverly, MA).

COS cell transfections

The cDNA encoding human fetal tau (352 residue isoform) cloned into the pRc/CMV mammalian expression vector, and fyn expression plasmid were used for transfections in COS cells as previously described (Lee et al., 1998). Cells were harvested 48 hours after transfection in a microtubule-stabilizing buffer (80 mM Pipes pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitors, 10 μM taxol and 1 mM GTP. Following a 15 minute incubation at 37°C, the lysate was centrifuged to pellet microtubules. The entire microtubule pellet from each 100 mm dish transfection was loaded. Microtubules were probed by western blotting using anti-phosphotyrosine (4G10) and anti-tubulin (DMA1, Sigma-Aldrich Co., St Louis, MO). Quantification of signal was performed by phosphorimaging (Bio-Rad Fluor-S Max MultiImager, Bio-Rad Laboratories Inc., Hercules, CA). It was noted that the transfections with Fyn had a reduced level of microtubules, possibly due to cellular toxicity caused by Fyn expression.

3T3 cell transfection, treatment and immunofluorescence

Tau transfections were performed with cDNA encoding the 352 residue isoform of human tau as previously described (Lee et al., 1998). Double mutants mimicking phosphorylation at Ser262 and Ser356 or at Thr231 and Ser235 were synthesized using site directed mutagenesis (Quik Change, Stratagene, La Jolla, CA) to convert Ser or Thr to Asp. Src transfections were performed with a mouse Src cDNA plasmid (Upstate USA, Charlottesville, VA). The constitutively active src cDNA was created by mutating Tyr529 to Phe. 3T3 cells, plated on glass coverslips, were transiently transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Transfected cells were stimulated with 50 ng/ml PDGF (Sigma-Aldrich, St Louis, MO) in serum-free medium. Cells were harvested at the times indicated. In some experiments, the cells were treated with 0.1 μM taxol or 10 μM SFK inhibitor PP2 (Calbiochem at EMD Biosciences, Pasadena, CA) at different time periods before or after PDGF stimulation.

Cells were fixed with 0.3% glutaraldehyde for immunofluorescence as previously described except with the use of 0.1% instead of 0.5% NP40 (Lee et al., 1998). For active Src labeling, the cells were fixed with 4% paraformaldehyde instead of glutaraldehyde. Antibodies used for immunofluorescence were polyclonal affinity-purified anti-tau [CR (Lee et al., 2004)], anti-Src-phospho-Tyr416 mouse monoclonal clone 9A6 (Upstate USA) and anti-acetylated tubulin (6-11B-1, Sigma-Aldrich). Actin was labeled with Phalloidin-Alexa 594 (Molecular Probes at Invitrogen, Carlsbad, CA). All secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Epifluorescence was performed using Nikon E800 microscope with a 60× Plan Apochromat objective (NA 1.4). Images were acquired using a Princeton Instruments Micromax CCD camera and Metaview software (v.4, Molecular Devices). Images were processed using Photoshop 7.0 (Adobe Systems).

Quantification and statistics

Following different treatments, over 100 non-transfected or tau-transfected 3T3 cells were examined per experiment. Cells were classified into two groups based on either the presence or absence of stress fibers (cells that had a few actin fibers remaining, with the majority of the actin filaments broken down, were classified as lacking stress fibers). A fraction of the cells (less than 15% of transfected as well as non-transfected cells) exhibited an intermediate morphology that could not be classified into either category; such cells were omitted from the analyses. Three independent experiments were performed for each experimental condition and the percentage of cells in each of the two groups was calculated. The results were subjected to statistical analysis using unpaired *t*-test.

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