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Phosphorylation Regulates Tau Interactions with Src Homology 3 Domains of Phosphatidylinositol 3-Kinase, Phospholipase Cγ1, Grb2, and Src Family Kinases*

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The microtubule-associated protein tau can associate with various other proteins in addition to tubulin, including the SH3 domains of Src family tyrosine kinases. Tau is well known to aggregate to form hyperphosphorylated filamentous deposits in several neurodegenerative diseases (tauopathies) including Alzheimer disease. We now report that tau can bind to SH3 domains derived from the p85α subunit of phosphatidylinositol 3-kinase, phospholipase Cγ1, and the N-terminal (but not the C-terminal) SH3 of Grb2 as well as to the kinases Fyn, cSrc, and Fgr. However, the short inserts found in neuron-specific isoforms of Src prevented the binding of tau. The experimentally determined binding of tau peptides is well accounted for when modeled into the peptide binding cleft in the SH3 domain of Fyn. After phosphorylation *in vitro* or in transfected cells, tau showed reduced binding to SH3 domains; no binding was detected with hyperphosphorylated tau isolated from Alzheimer brain, but SH3 binding was restored by phosphatase treatment. Tau mutants with serines and threonines replaced by glutamate, to mimic phosphorylation, showed reduced SH3 binding. These results strongly suggest that tau has a potential role in cell signaling in addition to its accepted role in cytoskeletal assembly, with regulation by phosphorylation that may be disrupted in the tauopathies including Alzheimer disease.

Tau is the principal microtubule-associated protein found in axons in the nervous system (1, 2) where it promotes the nucleation, elongation, stabilization, and bundling of microtubules (3). On cell fractionation tau is also found in non-cytoskeletal locations, and up to 25% of cellular tau has been reported to be in membrane fractions (4); it is also reported to be present in lipid rafts in neurons (5, 6) and oligodendrocytes (7).

Three or four partial repeats of 30–31 amino acids in the C-terminal half of tau, which show similarities to regions in MAP2 and MAP4 (8), provide most of the microtubule-binding affinity (see Fig. 1), whereas the N-terminal half of tau projects out from microtubules (9). These parts of the molecule may be involved in binding other proteins or cellular components (10), and tau has been shown to be able to bind to a variety of other proteins (for review, see Brandt and Leschik (11)), including a number that have roles in cell signaling.

Potential signaling proteins shown to bind both to microtubules and tau include protein phosphatases PP1 (12) and PP2A (13); manipulation of the binding of PP2A to tau affects its catalytic activity in cells (14). The scaffolding protein 14-3-3 binds to tau and is reported to mediate its binding to glycogen synthase kinase 3 (GSK3)[4] (15), an important tau kinase (16–19). Tau and arachidonic acid together activate phospholipase C (PLC) γ1 *in vitro* (20), and PLCγ1 can bind to tau in brain extracts (21).

The tyrosine kinases Fyn, cSrc, and Lck have been shown to bind to tau through their SH3 domains (22). SH3 domains normally recognize sites on proteins that contain the motif P*XX*P. There are seven P*XX*P motifs in human tau (see Fig. 1*B*), all of which include or are very close to known phosphorylation sites. Furthermore, when Fyn was co-expressed with tau in fibroblasts, the tau became phosphorylated on tyrosine residues (22), and phosphotyrosine-labeled tau has been found in tau from human fetal and Alzheimer brain (23, 24) as well as in brain from neonatal mice (25). In oligodendrocytes Fyn activity was required along with tau to allow process formation (7).

Although tyrosine phosphorylation of tau was discovered relatively recently, tau has long been known to be a phosphoprotein (26), with phosphorylation distributed over several ser-

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[4] The abbreviations used are: GSK3, glycogen synthase kinase 3; AD, Alzheimer disease; GAP, GTPase-activating protein; GST, glutathione *S*-transferase; PHF, paired helical filaments; PLCγ, phospholipase Cγ; HIV, human immunodeficiency virus; SH3, Src homology 3; NT, N-terminal; CT, C-terminal; CHO, Chinese hamster ovary.

ine and threonine sites (19, 27, 28). Tau isolated from the brains of fetuses or neonates is more highly phosphorylated than that from adults. Tau extracted from the brains of patients with Alzheimer disease (AD) is hyperphosphorylated, with phosphates distributed among 39 or more sites (19, 29, 30). Alzheimer tau is polymerized to form paired helical filaments (PHF) and straight filaments and aggregates of these filaments form neurofibrillary tangles, one of the two principal hallmarks of AD.

Hyperphosphorylated tau (PHF-tau) accumulates in brains before neurofibrillary tangles appear and is unable to bind tubulin or to promote microtubule formation or stabilization. However, when tau is enzymatically phosphorylated *in vitro*, for example by the mitogen-activated protein kinase ERK2, only a modest reduction in microtubule binding is apparent (31). The greatest effects on microtubule binding are given by phosphorylation within the microtubule binding repeat regions, whereas most of the phosphorylation sites are in the regions flanking the microtubule binding repeats (see Fig. 1*B*). Many of the serine and threonine residues that are phosphorylated both in normal tau and in PHF-tau are followed by prolines (19, 29, 30). Many of the functions of phosphorylation, therefore, are unknown, and it is unclear why so many sites can be phosphorylated even in normal (especially fetal) tau or what the causes and effects of hyperphosphorylation in the tauopathies might be.

It has been shown by co-immunoprecipitation that tau is bound to SH3-containing proteins in extracts from cultured cells (22, 23) and from brain (21) as mentioned above. We show here that tau can bind to several SH3-containing signaling molecules in addition to those already reported and that tau phosphorylation can reduce binding. Furthermore, model building based on extensive structural studies of SH3 domains provided useful insight into the putative molecular interactions and basis for binding to tau.

EXPERIMENTAL PROCEDURES

Plasmids for bacterial expression of human tau, isoforms 0N3R and 2N4R (32), and mutants of 2N4R tau with either one glutamate (S235E) or 18 glutamates in the N-terminal half of the molecule (ETau18, here referred to as Tau-E18) (33) were donated by Dr. M. Goedert and Dr. M. Smith (MRC Laboratory of Molecular Biology, Cambridge, UK). Tau-E18 had glutamates at the following positions: 46, 50, 69, 111, 153, 175, 181, 198, 199, 202, 205, 208, 210, 212, 214, 217, 231, and 235 (33). Tau with 10 glutamates (hTau441Glu10, *i.e.* based on 2N4R; referred to in this paper as Tau-Glu10) was donated by Dr. R. Brandt (University of Osnabrück) (34). Tau-Glu10 had glutamates at positions 198, 199, 202, 231, 235, 396, 404, 409, 413, and 422 (34) (numbering of the tau sequence is based on the 2N4R isoform of human tau, containing 441 amino acids (32)). Tau constructs for expressing the N-terminal (1–249) and C-terminal (250–441) halves of tau (Tau-NT and Tau-CT containing, respectively, residues 1–249 and 250–441 of 0N4R tau) were donated by Dr. H. Yanagawa (Keio University, Yokohama, Japan) (35). The various constructs used are illustrated diagrammatically in Fig. 1. All tau derivatives were expressed in *Escherichia coli* BL21(DE3) cells and purified as described previously (36, 37). Post-mortem brains from healthy and AD subjects were supplied by the MRC Neurodegenerative Diseases Brain Bank, King's College London Institute of Psychiatry. PHF-tau (30) and normal control brain tau (36) were purified as described previously in the presence of phosphatase inhibitors but omitting the chromatography stages. PHF-Tau was dephosphorylated by treatment with λ-phosphatase (New England Biolabs, Hitchin, UK) (38).

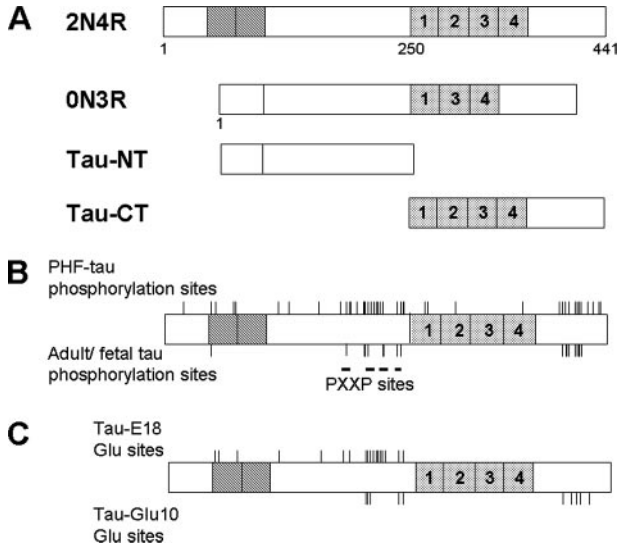

FIGURE 1. **Tau constructs used and positions of phosphorylation and pseudophosphorylation sites and proline-rich sequences.** *A*, tau isoforms and truncation constructs used. The variably spliced N-terminal exons are *shaded* (*left*), and the tubulin-binding partial repeats (*1–4*) are *shaded* and *numbered*. Repeat 2 is absent in 3R isoforms of tau. *B*, sites of phosphorylation determined by chemical or mass spectrometric sequencing (19, 29, 30), shown by *vertical lines*; those *along the top* have been found in PHF-tau, and those *along the bottom* have been found in normal adult and/or fetal tau; adult tau contains many of the same sites found in fetal tau but at lower stoichiometry (83, 84). Only the 2N4R isoform is shown, but human adult and PHF-tau consists of a mixture of all six isoforms, whereas fetal tau contains the 0N3R isoform. The positions of the four proline-rich regions, containing tau P*XX*P motifs, are shown as *thick lines*. *C*, the positions of serine and threonine residues replaced by glutamates are shown by *vertical lines along the top* for Tau-E18 and *along the bottom* for Tau-Glu10.

Constructs for bacterial expression of glutathione *S*-transferase (GST) (*i.e.* pGEX-4T empty vector) and fusion proteins with the SH3 domains of chicken cSrc, human Fgr, the p85α regulatory subunit of bovine phosphatidylinositol 3-kinase, human PLCγ1, and the C-terminal SH3 domain of human p67phox protein have been described previously (39–43). GST-SH3 constructs of human PLCγ2 and mouse Fyn were donated by Dr. S. Watson (Centre for Cardiovascular Sciences, University of Birmingham) (44) and Dr. S. Anderson (University of Colorado Health Sciences Center, Denver, CO) (45). Dr. I. Gout (Ludwig Institute for Cancer Research, UCL, London, UK) donated constructs for expressing neuronal Src with inserts of 6 or 17 amino acids (46), which are denoted here as nSrc6 and nSrc17, and purified GST-SH3 proteins with SH3 domains from Grb2 (both N- and C-terminal SH3s), p47phox (N-terminal SH3), and Ras-GTPase-activating protein (Ras-GAP) (39). Expressed recombinant proteins were purified on glutathione-Sepharose® beads according to the manufacturer's instructions (GE Healthcare).

To measure the binding of tau recombinant constructs to GST fusion proteins, 1–5 μl of glutathione-Sepharose beads

containing 2–10 μg of bound recombinant GST fusion protein were incubated at 4 °C with mixing by rotation with 0.2 ml of 10 μg/ml tau construct in binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.15 M NaCl, 0.5% (v/v) Triton X-100, 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1% (v/v) protease inhibitor mixture (Sigma, catalog no. P8849). After 1 h the beads were centrifuged for 1 min at 1500 × *g* at 4 °C. The supernatants were discarded, and the beads were washed twice in 0.75 ml of wash buffer (as binding buffer but without protease inhibitor mixture or serum albumin) and once in 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. Finally, 25 μl of 2× Laemmli sample buffer (47) was added to the beads (which had ~10 μl of residual buffer), and the tubes were vortexed and incubated for 5 min at 100 °C before being stored frozen for subsequent gel electrophoresis and Western blotting. A 5-μl sample of the binding solution (*i.e.* tau in binding buffer, as was used for incubating with beads as described above) was likewise treated with 25 μl of 2× sample buffer, heated, and stored frozen.

For expressing tau in mammalian cells, a V5-His-tagged construct in pcDNA3.1 was used as described previously (23). Constructs of hemagglutinin (HA)-tagged bovine p85α wild type and HA-p85α-ΔiSH2 in pEF vectors (48) were kindly provided by Dr. T. Mustelin (Burnham Institute, La Jolla, CA) and green fluorescent protein-p85α wild type (49) was kindly provided by Dr. W. Gullick (University of Kent). To express GST and GST-p85α-SH3 in mammalian cells, the pCEFL vector (50) was used. A pCEFL-GST-Net1 construct, kindly provided by Dr. A. Hall (Sloan-Kettering Memorial Hospital, New York), was used as template; QuikChange® mutagenesis (Stratagene, La Jolla, CA) was used to construct pCEFL-GST by introducing a stop codon before the Net1 coding sequence; pCEFL-GST-p85α-SH3 was constructed by insertion mutagenesis, inserting the coding region of p85α followed by a stop codon that had been generated by PCR from bovine p85α cDNA.

Chinese hamster ovary (CHO) and COS-7 cells were cultured, transfected, and harvested essentially as described previously (23). For transfection of CHO cells in 10-cm dishes, a total of 8 μg of DNA was used with 40 μl of Lipofectamine Plus reagent and 25 μl of Lipofectamine (Invitrogen). Cells were washed in Tris-buffered saline and harvested in lysis buffer consisting of 25 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.5% (v/v) Triton-X100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 150 mM NaCl, 10 mM NaF, and "Complete" EDTA-free protease inhibitors (Roche Applied Science). In some experiments Triton X-100 was replaced with 1% (v/v) Nonidet P40 (Igepal CA630, Sigma). For precipitation of GST or GST fusion proteins, lysate supernatants (0.6 ml) were incubated at 0–4 °C for 45 min with 5 μl of glutathione-Sepharose® beads (GE Healthcare) and then washed, treated, and analyzed as described above for cosedimenting recombinant proteins.

Peptides linked at their N termini to biotin via an aminohexanoic acid linker were synthesized by solid-phase procedures, purified chromatographically, characterized by both chromatography and mass spectrophotometry, and dissolved at 1 mg/ml in water. They were coupled to streptavidin-agarose beads (Sigma) by incubating with a 5-fold molar excess of peptide and 2 volumes of binding buffer. After mixing for 30–60 min, excess peptide was removed by washing with 2 × 0.75 ml of wash buffer. For binding to soluble GST-SH3 fusion proteins, 0.2 ml of binding buffer containing 15 μg/ml GST-SH3 or GST was incubated with 2.5 μl of peptide-loaded streptavidin beads for 1 h at 4 °C; the beads were then washed and treated with sample buffer as described above for glutathione beads. 1 μl of binding solution (GST protein in binding buffer) was added to 29 μl of sample buffer and heated and stored frozen as above.

Phosphorylated tau was prepared by incubating 66 μg of 2N4R tau with 0.75 μg of recombinant GSK3β (New England Biolabs) in a 50-μl reaction containing (final concentrations) 50 mM Tris-HCl, pH 7.5, 1.15 mM EGTA, 0.15 mM EDTA, 3 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 μM okadaic acid, 0.5% (v/v) protease inhibitor mixture (Sigma). After incubation for 6 h at 30 °C followed by 5 min at 100 °C, the sample was cooled and centrifuged, and the supernatant was stored at −20 °C. A control sample, without GSK3β, was prepared in parallel. For binding experiments to glutathione beads containing bound GST proteins, the buffer used was 40 mM β-glycerophosphate, pH 7.6, in place of 50 mM Tris in the binding and wash buffers.

Denaturing polyacrylamide gel electrophoresis and Western blots were performed as described previously (47, 51). The rabbit polyclonal anti-tau antibody used in most of the experiments was obtained from Dako Ltd (Ely, UK), whereas antibody TP007 against the N terminus of tau has been described previously (51). Anti-GST antibody was from GE Healthcare. Peroxidase-conjugated anti-goat antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were visualized by enhanced chemiluminescence (ECL) (GE Healthcare). In some experiments blots were visualized using secondary antibody coupled to IR800 dye (Rockland Immunochemicals), and fluorescence was scanned with the Odyssey system (Li-Cor Biosciences, Cambridge, UK). In Fig. 2 the images for blots or gels were cut and reassembled to remove unwanted lanes, indicated in the figures by *black vertical lines*. The panels shown contained lanes from the same gel or blot except in Fig. 6*B* where indicated.

Molecular modeling of peptide/SH3 binding was based on the peptide ligand interactions of Fyn SH3 in two known structures: (i) residues 91–104 of the p85α subunit of phosphatidylinositol 3-kinase, bound in the forward (class I) orientation (52) (Protein Data Bank code 1AZG); (ii) the HIV-1 Nef protein, bound in the reverse (class II) orientation (53) (Protein Data Bank code 1AVZ). The sequences of the tau peptide and the Fyn-SH3 ligand (1AVZ) or the p85α ligand (1AZG) were aligned and modeled in Quanta™. A set of alignments was generated to maximize the match of the proline residues of the tau peptide to the conserved proline residues of the 1AZG or 1AVZ ligands (to conserve the P*XX*P pattern). Furthermore, if an arginine or a lysine were present, then the peptide was aligned such that the basic residue of the tau peptide matched with the arginine in the 1AZG or 1AVZ ligands. The spatial coordinates of the template peptides were then adopted by the various aligned peptides and modeled (43) to the SH3 domain. The template peptides were then deleted from the structure to give a set of template-tau peptide models. Each model was investigated visually to assess the likelihood of binding, and the most likely models were subjected to further modeling. To

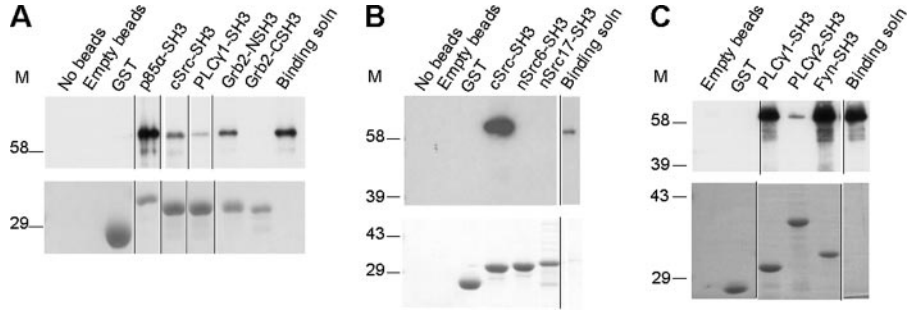

FIGURE 2. **Tau binding to SH3 domains.** GST-SH3 fusion proteins, attached to glutathione-Sepharose beads, were incubated with 2N4R tau, and co-sedimentation of tau was examined by denaturing gel electrophoresis and Western blotting. *Upper panels*, blots visualized with Dako anti-tau antibody using ECL; *lower panels*, Coomassie Blue staining of gels to demonstrate the presence and loading of the GST proteins. *A*, tau sedimented with SH3 domains from p85α, cSrc, PLCγ1, and Grb2 (N-terminal and C-terminal SH3 domains). *B*, comparison of cSrc-SH3 with neuronal-specific isoforms with inserts of 6 or 17 amino acids. *C*, comparison of PLCγ1, PLCγ2, and Fyn SH3 domains. *Binding soln* denotes the tau-containing solution used for incubating with beads. Blot and gel images had been cut and reassembled as shown by *black vertical lines*. Positions of molecular mass markers (*M*) are shown on the *left* (kDa).

TABLE 1
Summary of SH3 domains tested for binding to Tau

Protein	Binding	Reference
cSrc	+	(22), this work
nSrc6	−	This work
nSrc17	−	This work
Fyn	+	(22), this work
Fgr	+	This work
Lck	+	(22)
Abl	−	(22)
p85α	+	This work
PLCγ1	+	This work
PLCγ2	(+)	This work
Grb2-NSH3	+	This work
Grb2-CSH3	−	This work
Crk	−	(22)
p67phox-CSH3	−	This work
p47phox-NSH3	−	This work
Ras-GAP	−	This work
Spectrin	−	(22)
Amphiphysin	−	(7)

check the dynamic behavior of the modeled peptide, the peptide was regularized, and subsequently CHARM minimization and dynamics were performed.

RESULTS

SH3 Domains from Several Families of Signaling Proteins Bind to Tau—Fusion proteins of GST linked to SH3 domains from several proteins were tested for binding to recombinant 2N4R tau in co-sedimentation experiments with glutathione-Sepharose® beads. As shown in Fig. 2*A*, tau co-sedimented with SH3 domains of cSrc, PLCγ1, the N-terminal SH3 domain of Grb2, and most effectively with the p85α regulatory subunit of phosphatidylinositol 3-kinase. However tau did not associate with GST itself or with the C-terminal SH3 of Grb2. It also failed to associate with the neuronal-specific isoforms of Src, nSrc6 or nSrc17, which have inserts of 6 or 17 amino acids in their SH3 domains due to alternative splicing (46, 54, 55) (Fig. 2*B*). The SH3 domain of PLCγ2 also bound to tau, but the binding was much weaker than that of PLCγ1 (Fig. 2*C*). In other experiments (results not shown), tau was found to bind to the SH3 domain of the Src family kinase Fgr but not to those from p67phox (C-terminal SH3), p47phox (N-terminal SH3), or Ras-GAP. Results are summarized in Table 1 along with those reported by others. The shortest isoform of tau, 0N3R, also bound to the SH3 domains of p85α, Fyn, and PLCγ1 (results not shown).

Locations on Tau of Binding Sites for SH3 Domains—Tau contains seven P*XX*P motifs, which are all in the N-terminal half of the molecule (Fig. 1*B*). Lee *et al.* (22) showed that the SH3 domain from Fyn could bind to a truncated tau protein containing only the N-terminal half of the molecule but not to one containing only the C-terminal half based on three-repeat tau. Using the tau constructs Tau-NT (1–249 based on 0N tau) and Tau-CT (250–441 based on 4R tau), we confirmed that Fyn-SH3 could bind to Tau-NT but not to Tau-CT. We found also that Tau-NT, but not Tau-CT, was able to bind to the SH3 domains from p85α and PLCγ1 and from the Src family kinases cSrc and Fgr (results not shown).

To demonstrate unequivocally that P*XX*P motifs in tau are involved in SH3 binding reactions, peptides corresponding to each of the four proline-rich sequences were synthesized. Because the structures and binding properties of SH3 domains have been extensively described (52, 53), model building was used to guide in the selection of peptides as well as to predict possible outcomes of binding experiments.

Four peptides, numbered 1–4 (Fig. 3*A*), were initially chosen to span each of these four P*XX*P-containing regions. Fitting the peptides to the structure of the Fyn SH3 domain (45, 53) was attempted with the peptides oriented in either direction, *i.e.* class I or class II (56, 57). Results suggested that peptides 1 and 2 were unlikely to bind in either orientation, whereas peptide 3 (which conforms to a classical class II ligand, P*XX*P*X*R) and peptide 4 (which conforms to a classical Class I ligand, R*XX*-P*XX*P) both looked like potential candidates for binding but only when oriented, respectively, in reverse and forward directions. The predicted interaction of residues 215–225 of tau with the Fyn SH3 are shown in Fig. 4; these are modeled on the known structure of the HIV-1 Nef protein residues 71–81 bound in class II orientation to the Fyn SH3 domain. Furthermore, it was noted that if peptide 3 (which terminates at Pro-223) was extended to include Lys-224, then it might interact with Asp-99 in the Fyn SH3. Therefore, an extended version of peptide 3 was also synthesized (Peptide 3a). Variants of Peptide 3a were synthesized with Arg-221 replaced by alanine (Peptide 3b) or with prolines 216 and 219 both replaced by alanine, as these three residues are normally important for interaction of SH3 domains with class II ligands (57).

The biotinylated peptides shown in Fig. 3*A* were bound to streptavidin beads, and the binding of GST-SH3 fusion proteins was investigated using co-sedimentation assays (Fig. 3*B*). GST itself gave no significant binding to any of the peptides. Streptavidin beads without peptide or when loaded with Peptides 1 or 2 gave no significant binding to any of the fusion proteins. However, all three SH3 proteins tested (from p85α, Fyn, or PLCγ1)

gave detectable binding to Peptide 3, and in each case the binding was greater to Peptide 3a than to Peptide 3 (*i.e.* binding was improved by including lysines 224 and 225). Peptide 4 gave less binding than Peptide 3 but some binding was detected, particularly to the p85α SH3. Replacement of prolines 216 and 219 in Peptide 3a (Peptide 3c) drastically decreased the binding to all three proteins. Interestingly, replacement of Arg-221 (giving Peptide 3b) virtually abolished the binding of the SH3s from p85α and PLCγ1 but had relatively little effect on binding of the Fyn SH3; this suggests that lysines 224 and 225 can take over from Arg-221 in providing a binding partner. These residue specificities demonstrate that binding was as predicted for the active site of SH3 domains. This was confirmed by using a synthetic proline-rich peptide derived from p85α, residues 83–101, which is a known ligand for several SH3 domains (58); this blocked the binding of tau to the SH3 domains of p85α and cSrc in co-sedimentation experiments (results not shown).

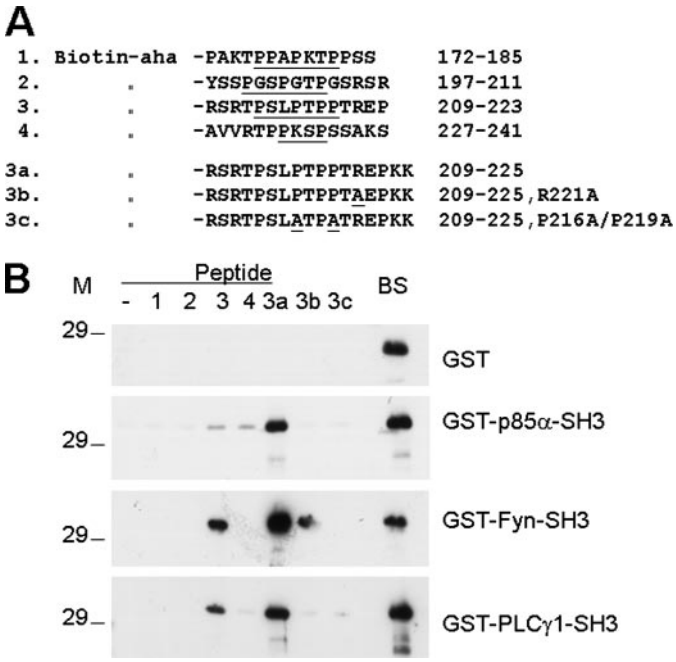

FIGURE 3. **Binding of SH3 domains to Tau-derived synthetic peptides.** N-terminally biotinylated peptides were synthesized as shown in *A*, with aminohexanoic acid linkers. For peptides *1–4* the P*XX*P motifs are *underlined*; peptides *1–3* each contain two P*XX*P motifs which share the central proline; hence, there are seven such motifs in all; for peptides 3b and 3c, the *underlined residues* are those altered from the native sequence present in peptide 3a. *B* shows Western blots, probed with anti-GST antibody and visualized by ECL; the peptides used for binding to streptavidin-agarose are shown *above each lane*; no peptide was used for the left-most lane. *BS*, binding solution containing soluble GST-SH3 protein. *M*, molecular mass markers.

Effect of Tau Phosphorylation on SH3 Binding—The results in Fig. 3 suggest that the SH3 domains from p85α, Fyn, and PLCγ1 bind to the regions around amino acids 213–221 (contained in Peptides 3 and 3a) and possibly also 230–236 (contained in Peptide 4). These regions contain or are very close to several known phosphorylation sites (19, 29, 30), including the known GSK3β phosphorylation sites Ser-210, Thr-212, Thr-217, Thr-220, Thr-231, Ser-235, and Ser-237 (19, 59–61). PHF-tau isolated from AD brain contains phosphorylation sites in these regions at Ser-210, Thr-212, Ser-214, Thr-217, Thr-231, Ser-235, Ser-237, and Ser-238 (19, 27, 29, 30, 62).

To investigate whether phosphorylation affects SH3 binding, tau was phosphorylated *in vitro* using recombinant GSK3β, and its binding was determined in GST co-sedimentation assays (Fig. 5*A*). The binding of all four SH3 domains tested (from Fyn, p85α, PLCγ1, and the N-terminal SH3 of Grb2) was considerably decreased by phosphorylation. However, the stoichiometry of phosphorylation by GSK3β *in vitro* is likely to have been only partial at any given site (also indicated by the presence of more than one band in the phosphorylated tau sample: Fig. 5*A*, *right panel*, *binding soln lane*), suggesting that only a partial reduction in binding would be detected, as was indeed the case.

To avoid problems of partial stoichiometry and uncertainty of the positions of the modified amino acids, pseudophosphorylation mutants were used with glutamate replacing one or more serines or threonines. Three such mutants were tested for binding to the SH3 domains from Fyn, p85α, and PLCγ1 (Fig. 5*B*). Replacement of Ser-235 alone by glutamate had little effect on tau binding to SH3 (Fig. 5*B*). In contrast, essentially no binding was detected for any of these SH3 domains to Tau-E18; this had 18 glutamates replacing serines and threonines in the N-terminal half of the molecule including all the sites that are followed by proline and also the known AD sites Ser-208, Ser-210, and Ser-214. Tau-Glu10, with 10 glutamates of which only five are in the N-terminal half of the molecule (Ser-198, Ser-199, Ser-202, Thr-231, and Ser-235),

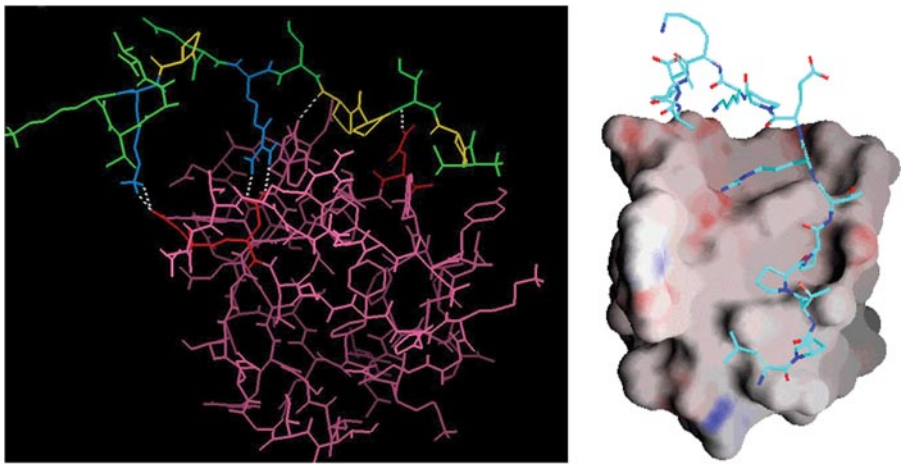

FIGURE 4. **Structure of the SH3 domain of Fyn binding to tau 215–225.** The three-dimensional structure of the Fyn SH3 domain with bound peptide from the Nef protein of HIV-1 (53) in class II orientation was used as the template for modeling interactions with peptide sequence from tau (see peptides 3 and 3a, Fig. 3). *A*, the SH3 structure is shown in *pink*, and the tau peptide is shown in *green*; the positively charged arginine and lysine are in *blue*, and prolines are in *yellow*. *B*, a Grasp surface model of the SH3 domain with the *red areas* representing the negatively charged surface of the domain, and *blue* representing the positively charged surface; the tau peptide is shown in a *stick representation*.

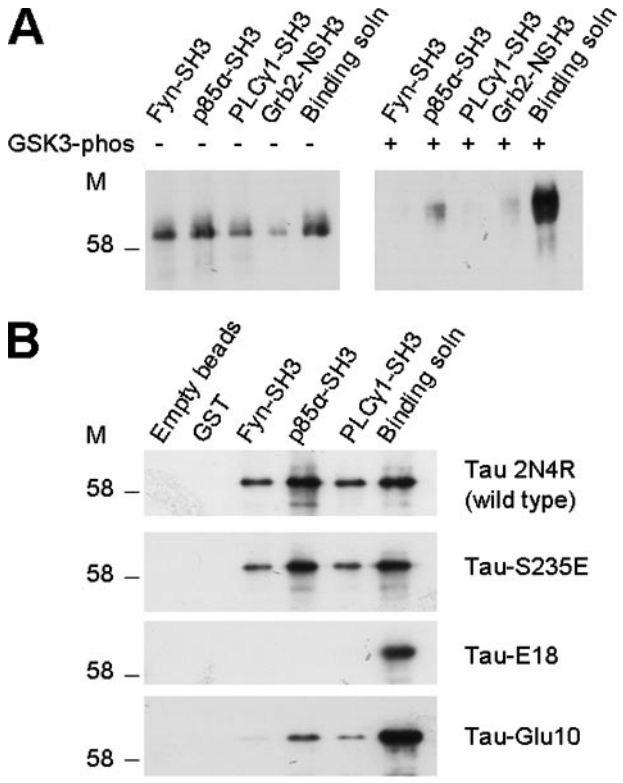

FIGURE 5. **Phosphorylation and pseudophosphorylation of tau inhibits binding to SH3 domains.** Cosedimentation analyses were conducted as in Fig. 2. *A*, GSK3β-phosphorylated tau (*right panel*) or control tau (*left panel*). *B*, wild type tau (*top panel*) and tau with glutamate replacing serine or threonine residues (*lower three panels*). Tau-S235E has glutamate replacing Ser-235, Tau-E18 has 18 replacements of Ser or Thr by glutamate in the N-terminal part of tau, and Tau-Glu10 has 10 glutamates replacing Ser or Thr, 5 in the N-terminal part of tau and 5 in the C-terminal part-see Fig. 1*C*. *M*, molecular mass markers.

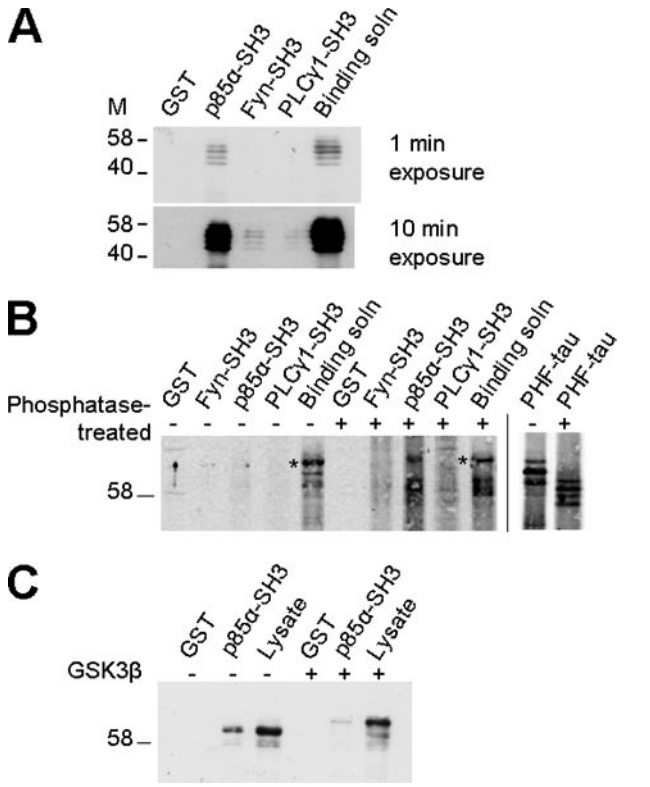

FIGURE 6. **SH3 binding of tau from brain and transfected cells.** Cosedimentation analyses were performed as in Fig. 2, with development of blots using ECL in *A* and *C* and with infrared fluorescence (Odyssey system) in *B*. *A*, tau isolated from healthy human brain. *B*, PHF-tau isolated from AD brain (*left-hand five lanes*) or dephosphorylated PHF-tau (*next five lanes*); the pair of lanes at the extreme *right* (from another gel) contain PHF-tau and dephosphorylated PHF-tau alone (without other components of the binding solutions). An *asterisk* marks a cross-reacting band in the binding solutions, and the *uppermost* of the *three main bands* in PHF-tau comigrated with this nonspecific band. *C*, lysates of tau-transfected COS-7 cells that had been co-transfected with pMT2 (empty vector) or GSK3β were incubated with glutathione-Sepharose beads containing GST or GST-SH3.

showed substantially reduced but still significant binding to p85α and PLCγ1 but little to Fyn (Fig. 5*B*). These results are consistent with the binding of SH3 domains to tau that is not phosphorylated (or glutamate-substituted) in the regions 213–221 and 230–236 and that phosphorylation (or glutamate substitution) within the region 210–220 and at 231 adversely affects binding. A plausible physiological function, therefore, for tau phosphorylation in these regions could be the regulation of binding of other proteins, with tau acting perhaps as a scaffold in cell signaling processes.

Tau from Cells Can Interact with SH3 Domains—To carry out a signaling role, tau in a cellular environment would need to be competent to bind to SH3 domains, and this binding would need to be regulated (inhibited) upon phosphorylation by active intracellular kinases.

Tau was isolated from normal healthy adult human brain and tested for binding to GST-SH3 fusion proteins by co-sedimentation (Fig. 6*A*). The SH3 domain from p85α showed good binding, and longer exposure of the blot showed that the SH3 domains from Fyn and PLCγ1 could also bind to brain-derived tau (Fig. 6*A*, *lower panel*). Examination of the different protein bands shows that the most slowly migrating bands gave less binding, whereas the faster-migrating bands bound preferentially, when compared with tau in the binding solution. This is consistent with stronger binding being given by less-phosphorylated tau components, which would migrate more rapidly. These results show that endogenous brain tau is competent to bind SH3 domains, especially p85α, and suggest that phosphorylation may reduce SH3 binding.

Because PHF-tau isolated from Alzheimer brain is highly phosphorylated, it might be expected on the basis of the above results to give much weaker binding to SH3 domains. As shown in Fig. 6*B*, PHF-tau was found to give scarcely detectable binding to the SH3 domains from Fyn, p85α, or PLCγ1. However, after dephosphorylation with λ-phosphatase, binding was restored, especially for p85α. The "PHF-smear" also showed binding to all three SH3 domains in addition to the tau monomer bands, although the latter were weak for Fyn and PLCγ1.

To examine the effects of tau phosphorylation in cells, COS-7 cells were transfected with tau-V5-His with or without co-transfection with GSK3β. As shown in Fig. 6*C*, binding to recombinant GST-p85α-SH3 was readily demonstrated (Fig. 6*C*, *second lane*), whereas no binding to GST was detected (Fig. 6*C*, *first lane*). Co-transfection of GSK3β decreased the amount of tau that bound to the beads (*lane 5*), and the decreased mobility of the tau band demonstrated that intracellular phosphorylation had occurred. Similar results were obtained using CHO cells. These results demonstrate that tau expressed in mammalian cells can readily bind to p85α-SH3, and phospho-

rylation by endogenous kinases is not sufficient to prevent binding. Increased intracellular GSK3 activity, however, largely prevented the binding, demonstrating the feasibility of regulating the SH3-tau interaction.

DISCUSSION

Although the C-terminal half of tau contains its microtubule-binding sites and is principally responsible for the tau microtubule-stabilizing properties, the function of the N-terminal half of the molecule was for a long time largely unknown (63). Tau has been suggested to be involved in cell signaling (see the Introduction). Phosphorylation of tau weakens its binding to microtubules and reduces its ability to promote microtubule assembly (31, 64–66), and hyperphosphorylated PHF-tau isolated from AD brain is essentially unable to enhance microtubule assembly (67); however, the large number of phosphorylatable sites (at least 15 in fetal and adult tau (19, 28, 29, 68) and up to 45 in PHF-tau (19, 30)) suggest that individual phosphorylation sites or groups of sites may have other more specific roles. The results presented here strengthen the case for tau being involved in cell signaling in several important respects.

Tau Binds to SH3 Domains from Several Types of Signaling Protein—Fyn and the related kinases cSrc and Lck have been shown to bind to tau through their SH3 domains (22). We found that the SH3 domain of Fgr also binds well to tau but that SH3 domains from the neuronal-specific Src isoforms nSrc6 and nSrc17 were unable to bind to tau. This suggests that these neuron-specific isoforms have distinct binding properties and functions (69).

However, SH3-tau interactions are not confined to Src family kinases. The interaction between PLCγ1 and tau, which activates PLCγ1 in the presence of arachidonic acid, has been suggested to involve the SH3 domain of PLCγ1 (20, 21), and we have now shown experimentally that this is the case. We also found that the SH3 domain of PLCγ2 binds only weakly to tau, consistent with PLCγ2 being confined to non-neuronal cells.

Furthermore, the SH3 domains of p85α (the regulatory subunit of phosphatidylinositol 3-kinase) and the N-terminal SH3 of Grb2, which are both important proteins for regulating cell growth and division, bind effectively to tau. This extends, therefore, the repertoire of established tau-SH3 interactions, which now includes four important types of signaling molecule. Table 1 summarizes data from this and earlier studies on SH3 binding to tau.

Studies Based on Structural Modeling Confirm Typical SH3 Interactions—Model building predicted that the sequence containing Peptide 3 would fit well to the SH3 domain of Fyn (Fig. 4). Co-sedimentation studies using immobilized synthetic tau-derived peptides (Fig. 3) demonstrated that Peptide 3 (containing the P*XX*P motifs at 213–216 and 216–219) did indeed bind to SH3 domains, and this was enhanced (as predicted by modeling) by extending the peptide to include Lys-224 (Peptide 3a). Alanine substitution of prolines 216 and 219 drastically reduced binding, whereas alanine substitution of Arg-221 almost abolished it for all except Fyn, where modeling suggested that lysines 224 and 225 may be able to take over the role of Arg-221. These results showed that the binding has the characteristics expected for peptide interaction with the active site of SH3 domains.

The deletion analysis of Lee *et al.* (22) had pointed to 233–236 as being a Fyn-binding site, but it did not reveal significant binding to the 213–219 region. Conversely, using synthetic peptides, we found binding to the 213–219 region but detected little binding to the 233–236 region. However, our Peptide 4 (Fig. 3), which included P*XX*P 233–236, was found to bind relatively poorly. Zamora-Leon *et al.* (70) used a peptide based on this region but with an extended N terminus that included lysines 224 and 225, and they successfully demonstrated binding to the Fyn SH3 in an enzyme-linked immunosorbent assay. Therefore, our results along with those of Lee *et al.* (22) and Zamora-Leon *et al.* (70) together suggest that both the 213–219 and 233–236 proline-containing regions are likely to be able to bind the SH3 domains of Fyn. The SH3 domain of p85α showed rather weak binding to Peptide 4 (containing 233–236) as well as to Peptide 3 (containing 213–219), whereas the SH3 of PLCγ1 binds, at least, to 213–219. The binding to glutamate-containing "phospho-mutants" (see below) suggests that each of these three SH3 domains may be able to bind to both 213–219 and 233–236. It appears, therefore, that SH3 domains that bind to tau are able to do so by using both 213–219 and 233–236, and the lysines at 224 and 225 may be important for both, suggesting mutually exclusive binding. The detailed preferences differ, however, for different SH3 domains.

Phosphorylation Inhibits Tau-SH3 Interactions—The presence of phosphorylation sites very close to the P*XX*P motifs in tau suggested that phosphorylation might regulate binding (20). The SH3 of Fyn bound preferentially to a basic component of tau from neuroblastoma cells (22), and PLCγ1 bound to a faster-migrating tau isoform in cell extracts (21); both of these results are consistent with preferential binding by less-phosphorylated forms of tau. In both PC12 cells (4) and SH-SY5Y cells (71) the tau that bound to plasma membranes was less phosphorylated than cytoplasmic or total tau. The direct demonstration here that phosphorylation of tau decreases its binding to SH3 domains establishes the reality of the effects of phosphorylation on SH3 binding. The absence of binding to SH3 domains by highly phosphorylated PHF-tau and its restoration by phosphatase treatment suggests that hyperphosphorylation could prevent the participation of tau in this aspect of cellular signaling.

Glutamate-containing phospho-mutants proved in this study, as in previous ones (4, 33, 34, 72, 73), to be a useful model of phosphorylation for determining the relevance of different sites. The lack of effect of the single substitution at Ser-235 is compatible with the peptide binding experiments of Zamora-Leon *et al.* (70), who showed that phosphorylation at Ser-235 had no effect on binding to the SH3 domain of Fyn, whereas in contrast, phosphorylation at Thr-231 prevented binding. Therefore, we tentatively interpret our results with Tau-Glu10 as follows; binding in the 231–236 region would be prevented by the glutamate at 231 (but not by the one at 235), accounting for the substantial drop in binding in this mutant, whereas the residual binding that is still found can be attributed to binding to the 213–219 region, which is unsubstituted. The same effect was seen qualitatively with the SH3 domains from Fyn, p85α, and PLCγ1, suggesting that all three are able to bind to each site, *i.e.* 233–236 and also 213–219, although to varying extents.

Phosphorylation at Thr-231 or glutamate substitution of this residue has been shown to have extensive effects on tau conformation (74), which may contribute to mediating the effects of modifying this site on SH3 binding. An equivalent threonine in MAP2c can be phosphorylated, leading to abolition of binding to Fyn-SH3 (70). Threonines 212 and 217 are also phosphorylation sites for GSK3β (61), and their phosphorylation may be responsible for inhibiting SH3 binding to the 213–219 region.

Bhaskar *et al.* (75), using surface plasmon resonance, found considerable differences in the binding of Fyn-SH3 to the 3R and 4R isoforms of tau that were, however, not shown by Src-SH3. Binding was generally weaker when Thr-231 and Ser-235 were mutated to aspartates; however, replacing Ser-199 and Ser-202 with aspartates caused larger changes, with increases in affinity of Fyn-SH3 for 4R tau but decreases for 3R tau. This is unexpected, because the Fyn-SH3-binding sites appear to be further downstream at residues 213–219 and 230–236 (Fig. 3 and Ref. 22). The differences between isoforms and tau mutants were tentatively attributed to conformational effects (75). However, all the tau forms used in the study by Bhaskar *et al.* (75) contained no N-terminal inserts, whereas we used constructs based on tau 2N4R for investigating the effects of phosphorylation. We did find, however, that tau 0N3R could bind well to the SH3 domains of Fyn, p85α, and PLCγ1 (results not shown). The point mutation R5H (Arg-5 to His, a frontotemporal dementia mutation) near the N terminus of tau produced considerable changes in binding to Fyn-SH3 (75), and therefore, the presence of N-terminal inserts could well induce conformational differences as well. Further work will be needed to explore the roles of phosphorylation in directly blocking SH3 binding and in mediating changes in binding via possible changes in conformation, with the latter perhaps being specific to particular SH3 domains.

Tau from Cells Binds to SH3 Domains and Is Phosphorylation-sensitive—It is already known that tau forms co-immunoprecipitating complexes with SH3-containing proteins in cultured cells and brain (21–23). Phospholipase Cγ1 could be co-immunoprecipitated with tau from rat brain extracts (21), and Fyn was co-immunoprecipitated with tau from extracts of SH-SY5Y neuroblastoma cells (22). Both Fyn and cSrc could be co-immunoprecipitated with tau when co-expressed in CHO cells (23). The potential binding, therefore, of tau to SH3 domains in cells and brain tissue is already well established. We wished to confirm that binding is indeed to SH3 domains and to determine whether phosphorylation of tau regulates this binding *in vivo*. We showed that tau in lysates from transfected cells is able to bind to the SH3 domain of p85α and that intracellular phosphorylation reduced this effect (Fig. 6*C*). We also showed that tau isolated from normal human brain is able to bind SH3 domains (Fig. 6*A*), whereas PHF-tau from Alzheimer brain is not (Fig. 6*B*). Therefore, tau expressed in mammalian cells has the properties required to bind intracellularly to SH3 domains in a regulated manner. Experiments that attempted to demonstrate co-sedimentation of GST-p85α-SH3 and tau, when both had been co-expressed in CHO cells, gave faint tau bands in some experiments but not in others, and co-immunoprecipitation of tau with p85α was also attempted unsuccessfully. This may be for methodological reasons; we observed that glutathione beads bound several endogenous cellular proteins and that capture of transfected GST proteins was only partial. Another possibility is that much of the cellular tau may be sequestered in the cell, *e.g.* by binding to microtubules or membranes, but is released on lysing the cells.

The reduction in binding to SH3 domains when tau is phosphorylated suggests that phosphorylation could participate in a signaling role that tau may have. It has long been noted that many of the serine and threonine phosphorylation sites in tau are followed by prolines (68). It now seems that in some cases it is the prolines that may possess the primary function (as key components of binding motifs) and that a function of phosphorylation at nearby residues may be to regulate this binding.

Phosphorylation of tau alters its binding to other proteins too. Tau has two binding sites for the scaffolding protein 14-3-3ξ, one in the microtubule binding region and a stronger one that is dependent on phosphorylation of tau on Ser-214 (see Ref. 76). Glutamate-containing phospho-mutants of tau showed markedly decreased binding to PP2A and to cell membranes (34, 72). In contrast, the WW domain of the prolyl isomerase Pin-1 binds to the 231–236 region of tau only when Thr-231 is phosphorylated (77, 78). Therefore, the same proline-rich region of tau can bind one protein when phosphorylated and others when not phosphorylated.

The results presented here show that tau has the potential for a broad role in cell signaling through binding to several important signaling molecules in a manner that is regulated by phosphorylation. Tau and Fyn are emerging, too, as mediators of neurotoxicity caused by amyloid β peptide (79, 80), with both being recruited to lipid rafts on exposure of neurons to amyloid β (6). This may involve excitotoxicity (80) or by stimulation of *N*-methyl-D-aspartate receptors (81); in the latter study N-terminal regions of tau were implicated. Complexes involving *N*-methyl-D-aspartate receptors, Fyn and PSD-95 that include tau have been suggested (82). Further experiments are needed to show whether tau signaling, tau-SH3 binding, and abnormal phosphorylation are involved in neurotoxic signaling and the development of AD pathology as well as in tau normal physiological function.

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Phosphorylation Regulates Tau Interactions with Src Homology 3 Domains of Phosphatidylinositol 3-Kinase, Phospholipase C γ1, Grb2, and Src Family Kinases

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