Interaction of Tau with the Neural Plasma Membrane Mediated by Tau’s Amino-terminal Projection Domain

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Abstract. The neuronal microtubule-associated protein tau is required for the development of cell polarity in cultured neurons. Using PC12 cells that stably express tau and tau amino-terminal fragments, we report that tau interacts with the neural plasma membrane through its amino-terminal projection domain. In differentiated PC12 transfectants, tau is found in growth cone-like structures in a nonmicrotubule-dependent manner. In hippocampal neurons, tau is differentially extracted by detergent and enriched in the growth cone and the distal axon when membrane is left intact. In PC12 transfectants, overexpression of tau’s amino-terminal fragment, but not of full-length tau, suppresses NGF-induced process formation. Our data suggest that tau’s amino-terminal projection domain has an important role in neuritic development and establishes tau as a mediator of microtubule-plasma membrane interactions.

Eukaryotic cells depend on microtubules for a variety of functions. In neuronal cells, a collection of specific microtubule-associated proteins (MAPs)1 are thought to aid microtubules in achieving their functional diversity and to have important roles in neuronal development (for reviews see Burgoyne, 1991; Tucker, 1990). The neuronal MAPs have distinct spatial localization in situ and are required for specific steps of process formation and differentiation. Suppression of the high molecular weight MAP (MAP2) inhibits minor neurite formation (Cáceres et al., 1992) whereas suppression of tau inhibits the development of axon-like processes in neuronal culture (Cáceres and Kosik, 1990), suggesting that tau has a key role in the establishment of neuronal polarity.

Tau consists of a family of closely related phosphoproteins whose expression is developmentally regulated and parallels neurite outgrowth (for recent reviews see Hirokawa, 1994; Lee, 1993). In situ, tau is primarily localized to the axon (Binder et al., 1985; Brion et al., 1988; Kowall and Kosik, 1987; Trojanowski et al., 1989). Overexpression of tau in cells of neural origin results in a more rapid extension of neurites and an increased stability of neuritic microtubules (Esmaeli-Azad et al., 1994). Functional characterization of tau fragments has revealed that the carboxyl-terminal end of the protein, which contains the microtubule-binding domain and a flanking region required for microtubule nucleation, is sufficient to promote microtubule assembly in vitro (Brandt and Lee, 1993) and to stabilize cellular microtubules (Lee and Rook, 1992; Léger et al., 1994). However the function of tau’s amino-terminal domain is poorly understood. This domain whose sequence is unique to tau, is subject to alternative splicing during neuronal differentiation. When tau is bound to microtubules, this domain protrudes from the microtubule surface about 19 nm (Hirokawa et al., 1988) which makes this projection domain a candidate for mediating interactions with other neuronal components. While there is evidence that tau interacts with components other than microtubules (Carlier et al., 1984; Correas et al., 1990; Griffith and Pollard, 1978; Loomis et al., 1990; Lu and Wood, 1993; Papasozomenos and Binder, 1987; Rendeon et al., 1990; Sattilaro et al., 1981) these interactions have not been mapped to the amino-terminal domain and their functional significance remains unclear.

The PC12 rat pheochromocytoma cell line has proven to be a powerful tool in studying neuronal differentiation (Greene et al., 1991). After exposure to NGF, PC12 cells acquire a number of properties characteristic of sympathetic neurons in that they cease proliferation and grow long neurites. NGF-induced PC12 show a transition from immature to mature tau isoforms (Hanemaaijer and Ginzburg, 1991) and tau expression parallels neurite outgrowth (Drubin et al., 1985). As a cell line, PC12 has a high degree of homogeneity important both for functional studies and biochemical analysis. In addition, protocols have been developed for efficient gene transfer into PC12 cells, facilitating the generation of stable cell lines.

We have studied the role of tau’s amino-terminal domain using PC12 cells that overexpress tau and tau fragments. We report that full-length tau and amino terminal...
fragments localize to the plasma membrane in these cells. Confocal immunofluorescence microscopy, flow cytometry, immunoelectron microscopy, and subcellular fractionation indicate that tau binds to component(s) at the cytosolic face of the plasma membrane. The membrane association of tau is also apparent in differentiating PC12 cells where tau is found in growth cone-like structures in a microtubule-independent manner. In cultured rat hippocampal neurons, membrane associated tau is predominantly found in the distal axon, and axonal growth cone. Upon NGF treatment, clonal lines of PC12 overexpressing amino-terminal fragments of tau fail to form neurites. Our data suggest that the association of tau’s amino terminal domain with the plasma membrane constitutes an important step in neuritic development and establishes a role for tau in mediating microtubule–plasma membrane interactions.

**Materials and Methods**

**Materials**

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and products for cell culture were obtained from GIBCO-BRL (Gaithersburg, MD) unless otherwise stated.

**Cell Culture**

PC12 cells were grown in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C and 10% CO2. For Geneticin-resistant clones, 250 μg/ml Geneticin were added. For NGF treatment, cells were seeded at a density of 2 × 10^4 cells/cm² on polylysine-coated surfaces (prepared by incubating with 100 μg/ml poly-D-lysine in 63 mM borate buffer, pH 8.5, for 5 h at 37°C) in DMEM containing 1% serum (0.67% fetal bovine serum and 0.33% horse serum) and antibiotics. The next day, 75 mouse NGF was added to 100 ng/ml. For preparation of 1-3 labeled cells, PC12 cells were washed once with labeling medium (minus-methionine-DMEM, supplemented with 10% diazylated fetal bovine serum and 5% diazylated horse serum) and incubated with 500 μCi L-[^35]S-methionine (Amersham Buchler GmbH, Braunschweig, Germany) in 10 ml labeling medium per 15-cm TC-dish for 6 h at 37°C and 10% CO2.

**RAT-1 Cells**

RAT-1 cells were grown in DMEM supplemented with 5% fetal bovine serum and Antibiotics. Rat hippocampal neurons were prepared and grown in low-density culture according to standard procedures (Goslin and Banker, 1991) except some modifications. In brief, hippocampi were dissected from brains of 18-20-old rat fetuses in 37°C Ca-Mg-free Hanks balanced salt solution (HBSS) supplemented with 0.6% (wt/vol) glucose and 7 mM Hepes, pH 7.4, treated with 0.25% trypsin for 15 min at 37°C, washed three times with HBSS, and dissociated by repeated passages through a fire polished Pasteur pipette. The cells were plated at a density of 8,000 cells/cm² on glass coverslips precoated with poly-D,L-ornithine and laminin (Boehringer Mannheim GmbH, Mannheim, Germany) and cultivated in MEM containing the NS2 supplements of Bottenstein and Sato (1979), 0.1% (wt/vol) ovalbumin, and 0.1 mM pyruvate. After 3 d in culture the cells were used for immunocytochemistry.

**Construction of Expression Vectors and Transfections**

Adult rat tau and two amino terminal fragments ending at nucleotide 605 and 688 respectively were prepared by PCR using an adult rat tau cDNA plasmid (Kosik et al., 1989) as template. Products were subcloned into pRcCMV (Invitrogen, San Diego, CA) and pRcCMV, a modification of pRC/CMV that expresses proteins with the sequence DYKDDDDK (FLAG; Hopp et al., 1988; Prickett et al., 1989) fused to the amino terminal end as an epitope tag (Léger et al., 1994). Tau[605] contained, in addition to the tau sequence, at its carboxyl terminal end the sequence LEG-PIV from the vector. The primary structure of FLAG-Tau[605] was validated by DNA sequencing. One conservative substitution (G-→V at position 198) was found. Fragments ending at nucleotide 605 and adult rat tau were obtained independently.

For establishing stably transfected clonal cell lines, PC12 cells were seeded 12–16 h prior to transfection at 5 × 10^4 cells on 10-cm culture dishes. Transfections were performed using 160 μl lipofectin and 30 μg DNA in OPTI MEM for 8 h. Transfections were performed with pRcCMV expressing the amino terminal fragments Tau[605] and Tau[688] and with pRcCMV containing full-length adult rat tau, Tau[605], and Tau[688]. As a control, transfection was also performed with pRcCMV containing no tau sequence. Selection with 500 μg/ml Geneticin was begun 5–7 d after transfection for a period of 14 d after which Geneticin was reduced to 250 μg/ml. As soon as individual clones could be identified, they were picked under the dissection microscope and transferred to collagen-coated 24-well plates. Confluent cultures were divided onto collagen-coated 24-well plates in duplicate. For immunoblot analysis, one set of the cultures was lysed with 50 μl RIPA (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% (vol/vol) NP40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS) and protease inhibitors (1 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, pepstatin, and trypsin inhibitor). Clones expressing high amounts of the particular construct as judged by immunoblot analysis using monoclonal anti-tau (Tau-1) antibody were amplified and frozen in aliquots. 20–70% of the Geneticin-resistant clones were found to express the transfected DNA. Geneticin-resistant clones obtained after transfection with control vector (pRcCMV) showed no immunoreactivity with an anti-FLAG antibody and individual clones were randomly selected.

For immunoblot transfections in RAT-1 cells were performed as in PC12 cells but with 10 μg/ml lipofectin and 5 μg/ml DNA in OPTI MEM overnight. Analysis was performed 2–3 d after transfection.

**Immunoblot Analysis and Immunoprecipitations**

Samples of lysate and subcellular fractions were boiled for 5 min in Laemmli SDS sample buffer and separated by SDS–polyacrylamide gel electrophoresis with 15% polyacrylamide (Laemmli and Favre, 1973). Transfer onto PVDF was overnight in 25 mM Tris, 192 mM glycine, 20% methanol at constant current (100 mA).

For colorimetric detection, blots were blocked for 2 h at room temperature in TS (10 mM Tris/HCl, pH 7.4, 0.9% [wt/vol] NaCl) containing 5% (wt/vol) BSA. Detection was with monoclonal anti-tau (Tau-1), anti-FLAG (M2; Kodak Corporation), or anti-α-tubulin (DM1A) antibody and alkaline-phosphatase coupled goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA) in TS containing 5% (wt/vol) BSA for 1.5 and 0.5 h (primary and secondary antibody, respectively). Blots were developed in 0.33 mg/ml nitro blue tetrazolium, 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 0.1 M carbonate-buffer, pH 9.8, 1 mM MgCl₂.

For detection by enhanced chemiluminescence (ECL), blocking was for 1 h at room temperature in TS containing 5% (wt/vol) non-fat dry milk (SmithKline), 0.05% (vol/vol) Tween 20. Detection was with monoclonal anti-tau (Tau-1), anti-FLAG (M2), and horseradish-peroxidase coupled goat anti-mouse secondary antibody (Amersham Buchler GmbH) in TS containing 0.05% (vol/vol) Tween 20 for 1.5 and 45 min (primary and secondary antibody, respectively). Blots were developed using the ECL system according to the manufacturer’s protocol (Amersham Buchler GmbH). For quanification, films were preflashed prior to exposure. Densitometry used a LKB Ultrascan XL Laser Densitometer.

For immunoprecipitations, 35S-labeled membrane fractions were solubilized with 0.3% (wt/vol) CHAPS in a total volume of 1 ml TS. Particulate material was pelleted for 10 min at 15,000 g and the supernatant incubated with 25 μl of normal mouse serum (Calbiochem-Behring Corp., San Diego, CA) for 1 h on ice then suspended in 25 μl Pansorbin cells (Calbiochem). After 30 min on ice, cells were pelleted for 15 min at 15,000 g. The supernatant was incubated with a mixture of two monoclonal anti-tau antibodies (Tau-1 and 5E2) for 1 h on ice then suspended in 10 μl protein g-agarose (Calbiochem-Behring Corp. After 1 h the precipitation was collected by centrifugation for 15 s at 15,000 g, washed four times with 0.3% CHAPS in TS, resuspended with 50 μl Laemmli SDS sample buffer, boiled for 5 min, and separated by SDS-PAGE on a 5–15% polyacrylamide gradient. The gel was prepared for fluorography using AMPLIFY (Amersham Buchler GmbH) according to the manufacturer’s protocol.

**Immunofluorescence and Immuno- electron microscopy**

For immunofluorescence, all cells were grown on polylysine-coated glass coverslips. For fixation without detergent extraction, cells were incubated with 4% (wt/vol) paraformaldehyde in PBS (10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl) containing 4% (wt/vol) sucrose for 20 min
at room temperature (RT). After washing with PBS, cells were incubated with 0.1 M glycine for 20 min and permeabilized for 90 s in 0.2%(vol/vol) Triton X-100 in PBS. For the combined NP-40 permeabilization-fixation protocol, cells were incubated in the presence of both 0.5%(vol/vol) NP-40 and 0.1%(vol/vol) glutaraldehyde as previously described (Lee and Rook, 1992); this protocol does not extract the cell but will preserve the association between tau and microtubules. For saponin extraction of cells, a protocol from Nakata and Hirokawa (1987) was adapted. Cells were washed in extraction buffer (80 mM PIPES/KOH, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 30%(vol/vol) glycerol, 1 mM GTP), incubated for 30 s with extraction buffer containing 0.2%(wt/vol) saponin, and washed with extraction buffer, with each step at 37°C. Cells were fixed for 1 h at room temperature with 2%(wt/vol) paraformaldehyde, 0.1%(vol/vol) glutaraldehyde in extraction buffer, washed with PBS, permeabilized with 0.1%(vol/vol) Triton X-100 in PBS for 30 min, rinsed with PBS, incubated for 7 min with 10 mg/ml NaBH4 in PBS, and 20 min with 0.1 M glycine in PBS. Following fixation, cells were blocked for 1 h with PBS containing 1%(wt/vol) BSA. For staining of plasma membranes, cells were fixed for 20 min with 2%(wt/vol) paraformaldehyde, 0.1%(vol/vol) glutaraldehyde in PBS, washed with PBS, incubated for 30 min with 10 µg/ml CM-DiI (Molecular Probes, Eugene, OR) on ice, washed with PBS, postfixed for 20 min with 2%(vol/vol) paraformaldehyde in PBS, incubated for 7 min with 10 mg/ml NaBH4 in PBS, and permeabilized for 90 s with 0.2%(vol/vol) Triton X-100 in PBS.

Staining was essentially as described earlier (Lee and Rook, 1992) in PBS containing 1%(wt/vol) BSA using monoclonal anti-tau (Tau-1, 461, 7A6), anti-FLAG (M5; Kodak Corporation), anti-MAP2 (HM-2), and anti-a-tubulin (DM1A) antibody, affinity purified tau polyclonal antibody (Pfeffer et al., 1983) and anti-tubulin antiserum, rhodamine-coupled donkey anti-mouse antibody and Texas red and 4',6-diamidino-2-phenylindole (DAPI) (Pfeffer et al., 1983) and anti-tau (Tau-1), horseradish-peroxidase coupled anti-mouse antibody, and enhanced chemiluminescence (ECL). Molecular weight markers are indicated at right. (C) Immunofluorescence staining for tau in PC12 clones, stably transfected with the constructs indicated. Staining was performed with monoclonal anti-FLAG (M5) (FLAG-Tau, FLAG-Tau[605], vector-control), or anti-tau (Tau-1) antibody (Tau[605]). Similar staining was observed with cells expressing FLAG-Tau[605] and Tau[605] (not shown). Bar, 10 µm.

Subcellular Fractionations

Cells from 10 confluent 15-cm cell culture dishes were transferred into a 50-ml Falcon-tube, washed twice with 40 ml PBS each and once with 5 ml 0.25 M sucrose by centrifugation for 5 min at 200 g. Cells were resuspended in 1 ml of 0.25 M sucrose containing protease inhibitors (1 mM EDTA, 1 mM PMSF, 10 µg/ml each of leupeptin and pepstatin), homogenized by 15 strokes with a tight fitting steel homogenizer and centrifuged for 5 min at 1,300 g. The pellet was washed twice with 0.25 M sucrose containing protease inhibitors and the initial supernatant and the wash solutions were combined for the post-nuclear supernatant (PNS).

Figure 1. Expression of tau fragments in PC12 cells. (A) Schematic of expressed tau sequence. The microtubule-binding domain is shown by the white box. Adult-specific sequence is indicated shaded (exon 2), black (exon 3), and hatched (exon 10). Some of the constructs contain an amino terminally fused epitope tag (FLAG), whose sequence is shown on the bottom. (B) Immunoblot of lysates of PC12-clones, stably transfected with the constructs indicated, after detection with anti-tau (lane 1) and anti-FLAG (lane 2) antibody. Expressed constructs and endogenous tau are indicated by the arrowheads and the arrows respectively. Electrophoretic separation was done by SDS-PAGE with 15% polyacrylamide. Transfer, immunoreaction, and detection were performed as described under Materials and Methods using monoclonal anti-FLAG (M2), anti-tau (Tau-1), horseradish-peroxidase coupled anti-mouse antibody, and enhanced chemiluminescence (ECL). Molecular weight markers are indicated at right.
Figure 2. Immunofluorescence staining after combined NP-40 permeabilization-glutaraldehyde fixation to reveal cytoskeletal association. Double staining for tau (left column) and tubulin (middle column), and staining for the FLAG epitope (right column) of PC12 clones, stably transfected with the constructs indicated, after combined NP-40 permeabilization-glutaraldehyde fixation. Note the presence of filamentous tau staining colocalizing with microtubules in cells expressing FLAG-Tau (top row) but not in cells expressing FLAG-Tau[605] or the vector control (middle and bottom row). Nonfilamentous, diffuse anti-tau staining was also observed in cells expressing the other amino-terminal tau fragments (not shown). Cells were grown, fixed and stained as described under Materials and Methods using affinity purified tau polyclonal antibody (Pfeffer et al., 1983), and monoclonal anti-a-tubulin (DM1A) and anti-FLAG (M5) antibody.

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Results
Expression of Tau and Amino-terminal Fragments in PC12 Cells

Stably transfected clonal lines of PC12 expressing full-length adult rat tau and amino-terminal fragments (Tau[605] and Tau[688]; Fig. 1 A) were prepared and isolated. The full-length tau sequence encoded the 432-residue rat adult tau isoform which contains the adult-specific exons 2, 3, and 10 (Kosik et al., 1989). The two amino-terminal fragments corresponded to the nonmicrotubule-binding projection domain with or without a putative weak microtubule interaction site (Tau[688] and Tau[605] respectively; Brandt and Lee, 1993). To allow identification of the expressed proteins, some of the constructs were epitope tagged using the FLAG sequence fused at the amino terminus. Clonal lines transfected with vector containing the FLAG but no additional tau sequence were prepared as controls. Fig. 1 B shows immunoblots of lysates of the established lines. Amino-terminal fragments had an apparent molecular weight between 41 and 44 kD, and full-length tau of 64 kD; all expressed protein ran at molecular weights higher than that expected from primary sequence, reflecting their extended structures (Cleveland et al., 1977). The level of expression was similar for all constructs as determined by quantitative immunoblotting and corresponded to an average of 6.9 pmol/10^6 cells, ranging from 4.0 to 11.8 for the individual lines. This amount was in the range of endogenous tau expression (7.1 pmol/10^6 cells in the vector control for the 55-66-kD isoforms). The amount of endogenous tau did not differ significantly in the different lines (8.6 pmol/10^6 cells ± 1.93 for the individual lines). In addition, the expression of the high molecular weight (120 kD) tau isoform (Couchie et al., 1992; Goedert et al., 1992) which is present in certain cell lines such as PC12 (Drubin et al., 1988) appeared to be unchanged. This indicates that the expression of the constructs...
Figure 3. Confocal image analysis of PC12 cells expressing tau fragments. (A and B) Confocal image analysis of typical cellular layers of PC12 cells expressing FLAG-Tau[605] (A), and FLAG-Tau (B). Double staining for the FLAG-epitope (left column) and the plasma membrane (middle column) in a longitudinal section, and anti-FLAG staining in a cross-section (right column) are shown. Similar tau distributions were observed in single-staining experiments using standard fixation with 4% paraformaldehyde (not shown). Note the colocalization of the tau fragments with the plasma membrane and the absence of tau staining from the site of attachment to the surface. (C) Confocal image analysis of a pair of PC12 cells expressing Tau[605]. Staining for tau is shown in a longitudinal section (left) and a cross-section (right) of the same cells. Note the absence of tau staining from the site of cell–cell contact. Cells were processed for immunofluorescence as described under Materials and Methods using monoclonal anti-FLAG (M5) (A and B) and anti-tau (Tau-1) (C) antibody, and the plasma membrane stain CM-DiI (A and B). Bar, 5 μm.

...did not alter endogenous tau expression. Expression of the fragment had no obvious effect on the morphology of the cells and did not affect the generation time (data not shown). It should however be noted that within a given clonal line, the level of expression was heterogeneous and varied between individual cells (Fig. 1 C). Therefore, the immunoblot results correspond to an average tau level expressed and individual cells may express significantly higher amounts of the construct than the immunoblot results indicate. In fact, quantitation of the level of expression from the intensity of immunofluorescent staining revealed that 24% of the cells expressed more than 1.5-fold and 16% more than 2-fold the mean level of FLAG-Tau. The heterogeneity of expression was stable over time and subcloning of high expressors was unsuccessful suggesting that the heterogeneous expression is an intrinsic feature of the particular clonal line rather than the result of an impurity. Heterogeneity of foreign gene expression in cell lines has been frequently observed however its reason remains unclear.

Association of Tau with Components of the Plasma Membrane

To test for the association of tau with the cytoskeleton, a
combined NP-40 permeabilization–fixation protocol known to preserve binding of tau to microtubules (Lee and Rook, 1992) was used. As expected, full-length tau was found to associate with microtubules whereas the amino-terminal fragments exhibited a nonfilamentous diffuse staining (Fig. 2), confirming that only expressed protein containing the microtubule-binding domain bind to microtubules under these conditions.

To analyze the subcellular localization of the constructs, transfected cells were fixed in the absence of detergent and examined by confocal immunofluorescence microscopy. All tau fragments were enriched at the periphery of the cell and colocalized with a plasma membrane stain (Fig. 3). The fragments were evenly distributed at the periphery but were absent from the surface attachment and cell–cell contact site (Fig. 3, A and B left, and C). To analyze the localization of tau further, a mild extraction protocol using saponin was used. This protocol selectively removes cytosolic proteins but retains cytoskeletal–membrane association (Nakata and Hirokawa, 1987). In saponized cells the amino-terminal fragment exclusively stained the cellular periphery (Fig. 4 A), indicating an association with plasma membrane components. The distribution was similar to the unextracted cells (Fig. 3 A). To analyze the localization at the ultrastructural level, immunoelectronmicroscopy of saponized cells was performed. In agreement with the immunofluorescence microscopic results, staining increased towards the periphery of the cell and was also observed directly adjacent to the plasma membrane (Fig. 4, B and C). To test for the presence of tau at the surface of cells flow cytometry was performed on living cells expressing tau fragments using monoclonal anti-FLAG, anti-tau, and, as a positive control, anti-rat MHC class I antibody (RT1A; Pharmingen, San Diego, CA). The cells showed no surface expression of tau indicating that tau is at the cytoplasmic face of the membrane (data not shown).

To test for a localization of tau to plasma membrane components in nonneuronal cells, a rat fibroblast cell line (RAT-1) was transiently transfected with tau fragments. As was the case in PC12 cells, FLAG-Tau associated with microtubules whereas FLAG-Tau[605] exhibited a nonfilamentous diffuse staining (Fig. 5, A and B). Confocal image analysis of unextracted cells showed no indication for an enrichment at the periphery of the cell (Fig. 5, C and D) suggesting that tau’s membrane-localization requires the presence of neural-specific factors. It should however be noted, that an enrichment at the membrane could have been difficult to observe due to the flat morphology of the cells.

To further analyze tau’s membrane interaction, subcellular fractionation of stably transfected clonal PC12 lines was performed. Fig. 6 A shows a schematic of the protocol employed, using low speed centrifugation of the cell homogenate to prepare a PNS and high-speed centrifugation to pellet membranes. The membrane fraction could be further processed by a high-pH wash to selectively remove peripherally bound proteins (Wiedenmann et al., 1985). As expected from the immunofluorescence microscopic results, tau was enriched in the membrane fraction as compared to the PNS or the soluble cytosol (Fig. 6 B). In contrast to tau’s fractionation with the membrane, tubulin was almost exclusively cytosolic, suggesting that interaction of tau with tubulin was not involved in tau’s membrane local-

Figure 4. Immunofluorescence and immunoelectronmicroscopy of saponin-extracted PC12 cells. (A) Anti-FLAG staining in a typical longitudinal section (left) and cross-section (right) of the same PC12 cell expressing FLAG-Tau[605] after saponin-extraction. (B and C) Immunoelectronmicroscopy of a saponin-extracted PC12 cell expressing FLAG-Tau[605]. C is a close-up of the region indicated in B. The nucleus (nu) is labeled. Note the enrichment of gold particles towards the periphery of the cell and their presence close to the plasma membrane as indicated by the arrow. Cells were processed for immunofluorescence or for immunoelectronmicroscopy as described under Materials and Methods using monoclonal anti-FLAG (M5) antibody. Bars: (A) 5 μm; (B and C) 100 nm.
Figure 5. Localization of transfected tau fragments in RAT-1 cells. (A and B) Double staining for tau (top) and tubulin (bottom) of RAT-1 cells, transiently transfected with FLAG-Tau[605] (A) and FLAG-Tau (B) after combined NP-40 permeabilization–glutaraldehyde fixation to reveal cytoskeletal association. Note the presence of filamentous tau staining colocalizing with microtubules in cells expressing FLAG-Tau but not in FLAG-Tau[605] expressing cells. (C and D) Anti-FLAG staining in a typical longitudinal section (top) and cross-section (bottom) of RAT-1 cells expressing FLAG-Tau (C) or FLAG-Tau[605] (D). Note that there is no obvious enrichment of either fragment at the periphery of the cell. Cells were transfected and prepared for immunofluorescence microscopy as described under Materials and Methods using affinity purified tau polyclonal antibody (Pfeffer et al., 1983) (A and B), monoclonal anti-α-tubulin (DM1A) (A and B), and anti-FLAG (M5) antibody (C and D). Bars: (A and B) 10 μm; (C and D) 5 μm.

out the FLAG-epitope. These tau deletion fragments (bands at 42 and 41 kD, respectively) similarly associated with the membrane fraction suggesting that the projection domain is responsible for tau's interaction with the membrane (Fig. 6, C and D). In agreement with the immunoblot results, the amino terminal fragment could be immunoprecipitated from solubilized membranes prepared from [35S]methionine labeled cells expressing FLAG-Tau[605] (Fig. 6 C). When probed with an anti-tau antibody, the immunoblots also showed anti-tau reactive species with molecular weights of 55–66 and 120 kD (Fig. 6 D). This indicates that all endogenous tau isoforms including the high molecular isoform are capable of associating with the membrane. This was confirmed by subcellular fractionation of wild-type PC12 cells (data not shown).

Interaction of Tau with Plasma Membrane Components in PC12 Neurites and Hippocampal Axons

To determine a possible membrane interaction of tau during process outgrowth, stably transfected PC12 cells expressing FLAG-Tau were analyzed after NGF exposure.
were cut after the detection, i.e., the intensity of the bands is directly comparable for the respective antibody. Note that FLAG-Tau (arrowheads) is enriched in the membrane fraction whereas the majority of tubulin (arrows) is in the soluble cytosol. (C) Immunoblot and immunoprecipitation of membrane fractions from a PC12 clone expressing FLAG-Tau[605]. For the immunoblot, sample preparation, blotting, and detection was as in B using monoclonal anti-FLAG (M2) antibody. For each lane, 50 μg of total protein were loaded. For the immunoprecipitation, membranes from [35S]methionine labeled PC12 expressing FLAG-Tau[605] were prepared and solubilized as described under Materials and Methods and precipitated in the absence or presence of a mixture of two monoclonal anti-tau antibodies (Tau-1, 5E2). (D) Immunoblot of membrane fractions from a PC12 clone expressing Tau[605]. Sample preparation, blotting, and detection was as in B using monoclonal anti-tau (Tau-1) antibody. For each lane, 100 μg of total protein were loaded. Note the presence of endogenous tau proteins (bands between 55 and 66 kD and at 120 kD; arrows) in addition to the transfected fragment (band at 42 kD; arrowhead) in the membrane fraction of FLAG-Tau[605] after detection with anti-tau antibody.

In contrast to wild-type PC12, tau expression in these cells was high enough to allow a high resolution immunofluorescence microscopic analysis of tau distribution. During differentiation, tau was associated with microtubules as judged by the NP-40 permeabilization–fixation protocol described above and was present at the periphery of growth cone-like structures suggesting a plasma membrane association in these regions (Fig. 7 A). Tau was not present in the actin-rich distal growth cone (Fig. 7 B) indicating that a direct interaction with actin filaments is not involved in tau’s localization. To test whether tau’s localization is dependent on the presence of intact microtubules, cells were saponin extracted under microtubule stabilizing and destabilizing conditions. Tau was present in the region of growth cone membranes under either condition (Fig. 7 C and D) indicating that intact microtubules were not required for tau to retain its membrane association. The results are consistent with a role for tau’s amino terminus mediating plasma membrane interactions in neurites.

Although PC12 cells grow neurites in a tau-dependent manner, they do not acquire axons and dendrites as do differentiated neurons. To determine tau’s interactions in axons, low-density cultures of rat hippocampal neurons, which represent a relatively homogeneous population of neurons with well-characterized properties (Goslin and Banker, 1991), were analyzed. After 3 d in serum-free medium, hippocampal neurons contain a fast growing axon in addition to shorter neurites which later develop into dendrites (stage III neurons; Dotti et al., 1988). In agreement with earlier reports (Dotti et al., 1987), tau was found in the soma and the axon when fixed without detergent extraction (Fig. 8 A). In saponin extracted neurons, tau was concentrated in the distal portion of the axon and the growth cone (Fig. 8 B and C). This distribution was confirmed by double staining saponin extracted neurons with anti-tau and anti-tubulin (Fig. 8, D and E). In saponin-extracted cells, identical tau staining patterns were obtained irrespective of the tau antibodies used (two monoclonals and two polyclonals tested). In the growth cone of saponin extracted neurons, tau had a filamentous distribution and colocalized with microtubules (Fig. 8, F and G). The pattern of staining suggests that bound tau and MAP2 localize to different microtubule populations in hippocampal neurons as they do in situ although total tau (i.e., bound and free tau) is not restricted to the axonal compartment in hippocampal cells.

Tau immunoreactivity was much reduced after extraction in the presence of 1% Triton X-100 (data not shown), suggesting a role for membranes in stabilizing tau’s binding to axial and growth cone microtubules. Together
epitope (left) and actin filaments (right) in FLAG-Tau expressing PC12 cells after combined NP-40 permeabilization-glutaraldehyde fixation. Note that tau staining is restricted to the proximal growth cone whereas actin filaments extend to the tips. (C and D) Double staining for the FLAG-epitope (left) and tubulin (middle), and phase-contrast image of saponin-extracted differentiated PC12 cells expressing FLAG-Tau. Extraction was either under microtubule stabilizing (C; extraction buffer with no Ca\textsuperscript{2+} and 1 mM EGTA) or destabilizing (D; +1 mM Ca\textsuperscript{2+}, no EGTA; 5 min extraction) conditions. Note that the tau staining at the periphery in growth cone like structures (arrows) persists whether or not the microtubules are destabilized. Cells were grown for 7 d (A and B) or 3 d (C and D) in the presence of 100 ng/ml NGF. Extraction, fixation, and staining was performed as described under Materials and Methods using affinity purified tau polyclonal antibody (Pfeffer et al., 1983) (A), monoclonal anti-\(\alpha\)-tubulin (DM1A) (A) and anti-FLAG (M5) antibody (B-D), and anti-tubulin antiserum (C and D). Double staining for actin filaments (B) was performed with FITC-coupled phalloidin.
with the data on the localization of bound tau, this supports the notion that tau binds to plasma membrane components which are specifically localized to these compartments.

Overexpression of Tau’s Projection Domain Suppresses NGF-induced Neurite Formation

To test for a possible function of tau’s amino-terminal domain in process outgrowth, the morphology of each stably transfected cell line was analyzed following NGF treatment. Control cells transfected with vector alone ceased proliferation, developed a flat phenotype, and generated neurites within some days after exposure to NGF as has been described for wild-type PC12 (Greene et al., 1991). Transfected cells expressing full-length tau, whether at high or low levels, responded to NGF similarly and acquired a flattened morphology and extended neurites (Fig. 9, A, D, and F). The only difference between these cells and control cells was an increase in the mean number of neurites per neurite bearing cell (Fig. 9 G). After 7 d of NGF treatment, this increase was significant (p < 0.05) and is consistent with a role of tau in neurite formation.

The morphology of high-expressing cells was evaluated for each stable transfectant after 3 and 7 d of NGF exposure, using two independent clonal lines for each construct. After 3 d of NGF, less than 25% of cells expressing the amino terminus (FLAG-Tau [650] and FLAG-Tau [688]) extended neurites (Fig. 9, B, C, and F). In contrast, cells expressing full-length tau were similar to control cells with the majority of cells (>75%) extending neurites (Fig. 9, A and F). The difference in cell morphology after 7 d of NGF was similar. It should be noted that cells expressing low levels of the amino terminus responded to NGF and extended neurites similar to control cells (77.4% ± 0.9% of cells expressing low amounts of FLAG-Tau[605] extended neurites after 7 d of NGF treatment; the subpopulation was selected by visual inspection of anti-FLAG stained cells and corresponded to about 20% of the total tau-expressing population) suggesting that the inhibition of neurite outgrowth was a function of fragment concentration. In cells expressing FLAG-Tau, tau was enriched at the periphery, both in the cell body and the neurites (Fig. 9 D). Cells which did not respond to NGF with neurite outgrowth exhibited fragment staining at the cellular periphery similar to the undifferentiated cells shown in Fig. 3 (Fig. 9 E). As mentioned above, cells expressing high levels of the amino terminus were morphologically indistin-
guishable from cells expressing high levels of full-length tau in uninduced PC12 cells, indicating that high levels of the amino terminus exerted a noticeable effect only during the differentiation process. The results suggest that the amino-terminal fragment suppresses neurite outgrowth by competing for plasma membrane binding.

Discussion

During neuronal development, microtubules must be assembled in a spatially and temporally defined manner in order to serve specific functions. In developing neurons, it is very likely that microtubule-associated proteins assemble and stabilize microtubules in specific cellular locations and help to organize microtubules relative to other cellular components. This is illustrated by the neuronal MAPs tau and MAP2. Both contain closely related microtubule binding domains at their carboxyl-terminal ends and have similar microtubule promotion activity in vitro. Yet, each has distinct functions during neuronal differentiation and localize to different microtubules in situ (MAP2 associating with somatodendritic microtubules and tau primarily with axonal microtubules). This suggests that their amino termini or projection domains, might each have specific unique functions that aid in creating functionally different microtubules. In this study we used genetic manipulation of PC12, an established and well-characterized cell line of neural crest origin, to determine the role of tau’s amino terminal projection domain. Our results indicate that tau interacts with components at the cytoplasmic face of the neural plasma membrane through its amino-terminal domain. This is based on our findings that: (a) the amino terminus localizes to the periphery of the cell when expressed in undifferentiated PC12 cells; (b) tau and amino-terminal fragments are present in the membrane fraction after subcellular fractionation and are sensitive to high-pH wash; and (c) in growing neurites, tau is present at the periphery of growth cone-like structures.

The functional significance of tau’s interaction with the membrane was investigated in cells overexpressing the amino-terminal domain of tau. While undifferentiated cells behaved similarly to control cells, cells exposed to NGF were deficient in neurite extension. The effect was most prominent in cells expressing high levels and suggested that high levels of the amino-terminal protein were capable of blocking the function of the endogenous tau, presumably by binding to specific components in the membrane and preventing full length tau from binding to the membrane. Indeed, in the few cells expressing high levels of the amino terminus that did extend neurites, immuno-fluorescence microscopy indicated that after saponin extraction, the fragment was absent from the neurites (data not shown). This suggested that displacement of the fragment from the membrane by NGF-induced endogenous tau accompanied neurite outgrowth in these cells. The quantitation of the expression levels of endogenous tau and the range of expression levels for the amino-terminal fragment indicated that comparable amounts of each protein are expressed, with possibly a molar ratio of 2 to 1 amino-terminal fragment to endogenous full-length tau in the high level expressors. Moreover, the membrane bound amino-terminal fragment would not interact with microtubules, suggesting that tau-mediated microtubule-membrane interactions are important for neuritic development. The importance of these interactions would not have been detected in tau antisense treated PC12 cells (Esmaili-Azad et al., 1994; Hanemaaier and Ginzburg, 1991) where the expression of tau was altogether inhibited. The analysis of PC12 cells overexpressing the amino-terminal fragment allowed the identification of a new tau function separate from its microtubule related activities.

We hypothesize that an integral or an intracellular membrane-bound protein exists which binds to tau, enabling tau to bridge microtubules to membranes. Moreover, tau would have a role in stabilizing microtubules close to membranes. The importance of this type of activity in neuronal development might be insinuated by the phenotype of mice deficient in tau expression. Strikingly, only small caliber axons of these mice had altered morphology and decreased microtubule stability (Harada et al., 1994). Since the surface to volume ratio in these axons is high, a large proportion of the microtubules would be close to the membrane. If tau had the specific role of stabilizing microtubules close to membranes, these axons would be severely affected by the depletion of tau. In addition, tau’s association with membranes may enhance its ability to stabilize microtubules in cells. Since the affinity of tau for microtubules is relatively weak as measured in vitro ($K_d = 10^{-7}$ M; Butner and Kirschner, 1991; Goode and Feinstein, 1994; Gustke et al., 1994), bringing membranes close to microtubules could limit tau’s diffusion away from microtubules thereby increasing its ability to stabilize microtubules in vivo.

The localization of tau to growth cone like structures in PC12 cells and rat hippocampal neurons raises the possibility that tau interacts with the growth cone membrane skeleton. This possibility has also been suggested by a recent study where rat cerebellar neurons treated with tau antisense had altered growth cone morphology (DiTella et al., 1994). In these cells, the localization of a putative actin-binding protein and the filamentous actin distribution in the growth cone were also disrupted, suggesting that tau might affect the microfilament network. While it is known that tau binds to and cross-links F-actin in vitro in a phosphorylation-dependent manner (Griffith and Pollard, 1978, 1982; Sattilaro et al., 1981; Selden and Pollard, 1983) the patterns of anti-tau and phalloidin staining in the growth cone were significantly different (data not shown) and tau was absent from the actin rich filopodia. Our data and that of DiTella et al. (1994) would suggest that tau in the growth cone associates with both microtubules and components in the membrane skeleton. The membrane association does not require microtubules and most likely involves tau’s projection domain.

Tau was first found enriched in axons in situ (Binder et al., 1985). However, a phosphorylation dependent antibody was used in this study (Tau-1) and subsequently, alkaline phosphatase treatment of the tissue before staining revealed tau in cell bodies and dendrites (Papasozomenos and Binder, 1987). While other axonal localizations of tau in situ have been reported using other antibodies (Brion et al., 1988; Trojanowski et al., 1989), it remains a controversial issue. Moreover, tau’s localization to the axon was not reproduced in primary cultured hippocampal, cerebellar,
FLAG-Tau(605)  
FLAG-Tau(688)  
FLAG-Tau  
Vector-control

Mean number of neurites:

3 days NGF  
7 days NGF

Cells with neurites (%):

F

G

FLAG-Tau(605)  
FLAG-Tau(688)  
FLAG-Tau  
Vector-control

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or whole brain neurons (Dotti et al., 1987; Ferreira et al., 1989; Litman et al., 1993) where tau was either ubiquitous or in the cell body in addition to the axon. Interestingly, by saponin extracting rat hippocampal neurons, the axonal specific localization of tau was reproduced (Fig. 8). This indicates that while tau is ubiquitous in cultured neurons, it can be differentially extracted with detergents. Using a mild detergent such as saponin preserves membrane-cytoskeletal associations and appears to enhance the ability of tau to remain bound to microtubules during the extraction protocol. Furthermore, since this microtubule bound tau is preferentially found in the distal axon, the axonal membrane domain might contain components specific for tau. These membrane components might contribute to the mechanisms by which tau is targeted to the axon. If this membrane domain existed in a vesicular form at some stage, newly synthesized tau would bind to these vesicles and the vesicles would be sorted to the axonal domain using mechanisms similar to those established for the intracellular trafficking of proteins in epithelial cells (reviewed by Rodriguez-Boulan and Powell, 1992). The site of this sorting would take place at the base of the axon where tau mRNA is preferentially localized (Litman et al., 1993).

Kanai and Hirokawa (1995) have suggested that the localization of tau to the axon is based on the microtubule binding properties of the carboxy terminal half of tau. However, in their system (17-d-old spinal cord neurons), neither endogenous tau or tau expressed by transfection appeared to be enriched in the distal portion of the axon or growth cone, with or without detergent extraction. Therefore, it is not clear whether our results can be compared. Moreover, it is conceivable that tau spatial localization and mechanisms used for its localization differ according to cell type and developmental stage.

The presence of tau in the axonal growth cone membrane skeleton early in development allows for speculation on the role of tau in signal transduction and the consolidation of growth cone movement. During PC12 differentiation, when NGF binds to its receptor at the cell surface, a specific sequence of events is triggered leading to cytoskeletal changes and neurite outgrowth. During this process, growth cones are established which contain very dynamic microtubules that transiently extend to the tips of neurites (DiTella et al., 1994; Tanaka and Kirschner, 1991). While growth cone guidance appears to be mainly actin-based, the selective stabilization of a subset of dynamic microtubules might lead to microtubule bundling and consolidation a transient orientation of the growth cone (Mitchison and Kirschner, 1988). Moreover, dynamic microtubules are required for the forward movement of the growth cone (Tanaka et al., 1995) and tau has been shown to co-localize to tyrosinated microtubules in the growth cone (DiTella et al., 1994). If tau associates with a member of the signal transduction cascade in the growth cone membrane, tau would then bind to dynamic growth cone microtubules whose orientation coincided with extracellular signals. These interactions could have a role in growth cone movement and process outgrowth.

As a next step towards understanding the role of tau's membrane association in developing neurites, it will be important to identify the interacting component(s) and to analyze its interaction with the cytoskeleton both in vitro and during neuronal development.

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