The Proline-Rich Domain and the Microtubule Binding Domain of Protein Tau Acting as RNA Binding Domains

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Abstract: Neuronal tau, through its proline-rich domain and the microtubule binding domain, binds to RNA non-sequence-specifically via electrostatic interaction. This binding inhibits the activity of tau. Tau and RNA were also found to co-localize in SH-SY5Y cells suggesting that RNA has opportunities to interact with tau in cells.

Keywords: Neuronal tau, RNA, non-sequence-specific RNA-binding, electrostatic interaction, proline rich domain, microtubule binding domain.

INTRODUCTION

Neuronal tau is a member of microtubule-associated proteins, promoting microtubule assembly and stabilizing microtubules [1-3]. This protein is a major constituent of paired helical filaments (PHF) found in Alzheimer's disease [4]. It was reported that some polyanions such as heparin and polyglutamate could induce filament formation [5]. Bryan *et al.* [6] proposed that protein tau is bound to RNA and the association inhibits microtubule assembly. Kampers *et al.* [7] and Hasegawa *et al.* [8] observed that RNA stimulates aggregation of protein tau into Alzheimer-like paired helical filaments. Ginsberg *et al.* [9] found that RNA is sequestered in the neurofibrillary lesions of Alzheimer's disease. Thus, it is interesting to investigate the mode of RNA-tau interaction.

Protein tau containing multidomains is a multifunctional molecule. It contains two major domains [10], a projection domain [11] and a microtubule binding domain (MTBD) [12]. The projection domain is composed of an acidic and a proline-rich region [10]. Kampers *et al.* [7] constructed twenty-one mutants of tau to investigate which domain is involved in the RNA-induced assembly of PHFs. They found that microtublule binding domain plays an important role in the interaction between tau and RNA. The three-repeat constructs polymerize most efficiently and two repeat constructs are the minimum number required for assembly. So far, no work has been done on the function of the proline-rich domain (PRD) in the association with RNA. Here, we report that protein tau binds to RNA non-sequence-specifically via the MTBD and the PRD through electrostatic interactions. Further study through indirect immunofluorescence assay suggests that RNA has some opportunities to interact with tau in cells.

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MATERIALS AND METHODS

Construction, Expression and Purification of Protein Tau and Its Mutants

Protein tau can be divided into four regions: the N-terminal region, the proline-rich domain, the microtubule binding domain and the C-terminal region [10-12]. To investigate the roles of these four regions in the interaction with RNA, we designed a group of primers to construct truncated tau (our unpublished data). These mutants are abbreviated as follows: τN (1-113), τPro (114-193), τMTBD (198-278) and τC (279-352). To further investigate the function of the MTBD, we constructed an MTBD-deleted mutant (ΔMTBD).

The constructs were prepared by PCR or megaprimer PCR amplification using htau-23 clones as the template. They were subcloned into the prokaryotic expression vector pET-28a(+) (Novagen, USA) as NocI / XhoI fragments. A hexa-his tag was fused to the C-terminus of these five mutants. The clones were transformed into *E. coli* BL21(DE3) strain after their nucleotide sequences had been confirmed by sequencing.

Proteins were expressed as described [3]. The purification of tau23 and tau40 was performed through the Q-Sepharose and SP-Sepharose chromatography (Amersham Pharmacia Biotech, Sweden) as described [3], while mutants were purified by a Ni-NTA column. Protein samples were run on gels and followed by staining with Coomassie brilliant blue. The protein bands on gels were verified by staining with anti-his tag monoclonal antibodies (Novagen) for tau mutants or anti-tau monoclonal antibodies (Santa Cruz) for protein tau23 or tau40.

Tubulin Purification and Assembly Assay

Tubulin was purified by phosphocellulose ion exchange chromatography of porcine brain extracts prepared by repeated cycles of assembly and disassembly as described [13]. The assembly condition and the turbidity measurement were

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referred to Kampers *et al.* [7]. Human tau40 (final concentration, 25 μg/ml) was used in this assay in 0.1 M MES Buffer (pH 6.4) containing 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM β-mer and 0.1 mM EDTA. The slope of the light-scattering intensity was used to estimate the activity of protein tau in the promotion of microtubule assembly.

Interaction of Protein Tau40 with RNA Assay

In tau-RNA binding experiments, 50 mM Hepes Buffer (pH 7.5, for tau23) or Pipes Buffer (pH 6.4, for tau40) containing 0.5 mM EGTA and 0.5 $MgCl_2$ was employed unless otherwise stated. Yeast total RNA (Amersham Pharmacia Biotech, Sweden), containing 0.5 U RNasin (Ribonuclease inhibitor), was incubated with protein tau40 at different concentrations. Incubation of tRNA or mRNA with protein tau was performed under the same conditions. Aliquots were run on 1% agarose gels. BSA and histone H2B were used as controls.

Analysis Binding of Protein Tau40 or Tau23 to Small RNA

$PolyA_{30}$ containing thirty adenosines and $PolyA_{15}U_{15}$ containing fifteen adenosines linked to fifteen uridines were synthesized by TAKARA Company (Japan). They were labeled with fluorescein at the 5' end. Synthesized RNA was incubated in the presence of protein tau40 or tau23 at 37 °C for 10 min. And then, aliquots were run on 8% non-denatured PAGE.

The Binding of Tau to RNA Observed by Atomic Force Microscopy

Protein tau40 (50 ng/μl) was incubated with tRNA or mRNA (10 ng/μl) at 37 °C for 15 min. Samples were diluted 5-10 times and then a 10 μl drop of the sample was deposited on freshly cleaved mica, allowed to stand for 5 min in air, and then washed with three aliquots (200 μl) of buffer solution before drying for 4 min in a stream of nitrogen. Tapping mode of atomic force microscopy (AFM) was performed using a Nanoscope IIIa Multimode-AFM (Veeco Instruments, USA) under ambient conditions. Silicon tips (TESP) with a resonance frequency of about 250 kHz were used at a scan rate of 1–2 Hz. Once the tip was engaged, the set point value was adjusted to minimize the force exerted on the sample while maintaining the sharpness of the image.

Analysis of the Charge Distribution of Protein Tau40

The method of Kyte and Doolittle was commonly used to analyze the hydrophobic characteristics of proteins through their primary structures [14]. Similarly, a window of 19 amino acid residues as a group to estimate the average charge distribution of protein tau40 is shown as the following equation.

$$Qi = (1 / 19) \sum_{j=i-9}^{j=i+9} qj$$

where $\mathbf{q_j}$ represents the charge of a amino acid residue at position **j**; $\mathbf{Q_i}$ stands for the average charge value for the amino acid residues from positions **i-9** to **i+9**.

Effects of Temperature, NaCl or pH on the Binding of Tau40 with RNA

Yeast total RNA mixed with protein tau40 was incubated at different pH values (2.6-12) or incubated with NaCl at various concentrations (0-1.0 M) at 37 °C for 10 min or were boiled for 0-20 min. To prepare the buffer (30 mM citric acid, 30 mM KH_2PO_4, 30 mM boric acid and 30 mM barbital) at different pH values, we adjusted pH with NaOH. Aliquots were run on agarose gels. Protein tau40 alone was used as control.

Analysis of Binding Tau Mutants to RNA

RNase A or DNase I was incubated with the purified tau mutants (τN, τPro, τMTBD, τC and ΔMTBD) from Ni-NTA column at 37 °C for 15 min. Aliquots were run on 1% agarose gels. Samples in the absence of nuclease were used as controls.

Indirect Immunofluorescence Assays of Protein Tau and RNA in Cells

Human neuroblastoma (SH-SY5Y) cells obtained from ATCC were grown in Dulbecco's modified Eagle's medium (DMEM) mixed with 10% fetal bovine serum (Gibco, USA) and 2 mM glutamine. For indirect immunofluorescence assays, cells were seeded onto cover slips at a density of $2x10^4$ cell/cm^2 in 24-well culture dishes and grown overnight at 37 ^oC in a humidified incubator with 5% CO_2.

SH-SY5Y cells were stained with 500 nM RNASelect green fluorescent cell stain (Molecular Probes, Invitrogen Inc, USA) in DMEM. And then, the cells were fixed in pre-chilled methanol at -20 °C for 10 min. After three washes with PBS, fixed cells were incubated with the labeling solution for 20 min at 37 °C followed by wash with PSB again. To label the endogenetic tau and inhibit the nonspecific antibody binding, we blocked the fixed cells with PBS contained 5% BSA for 30 min at 37 °C. Cells were permeabilized using 0.5% triton X-100 for 15 min and incubated with anti-tau antibody (Santa Cruz, CA) at 4 °C overnight. After three washes with PBS, the cells were incubated with the secondary antibody (Go-anti-Mo-IgG conjugated with TRTIC, Santa Cruz) at 37 °C for 30 min. For co-localization studies of RNA and protein tau in SH-SY5Y cells, the fluorescently labeled cells were visualized with an Olympus confocal microscope FV500 (FV500+IX81) using FITC and TRTIC filter sets.

RESULTS

Interaction of Protein Tau with RNA

Bryan *et al.* [6] reported that inhibition of tubulin assembly by RNA requires a protein. After the protein was sequestered by poly(A)-agarose and further investigated, they proposed that the protein is a tau-like factor. To further study the interaction of protein tau with RNA, we used human recombinant tau proteins. As shown in (Fig. **1A**), the light scattering intensity at 350 nm increased when protein tau was added to tubulin. The initial activity of tau, however, is markedly decreased in the presence of RNA. The activities decrease to about 66% (curve 3) and about 40% (curve 5) when the mass ratios between protein tau and RNA are 2:1

and 1:1. The microtubule assembly-promoting activity of tau protein in the absence of RNA was indicated as 100% under the same conditions (curve 1). Note that the amount of light scattering intensity did not change when the incubation of tubulin alone (curve 2) or protein tau40 with RNA in the absence of tubulin (curve 6) was used as control. Our results further confirm what Bryan *et al.* have reported, that is, RNA blocks the assembly of microtubules promoted by protein tau.

To detect whether the inhibition of protein tau promoting tubulin assembly by RNA could be relieved, we incubated protein tau with RNA at 37 ^{o}C for 60 min and then added RNase A (0.1 μg/ml) to the reaction mixture. As shown in (Fig. **1B**), aliquots were taken to measure the microtubule assembly-promoting activity. The activity of protein tau was reactivated when RNA was removed by RNase A (curve 4).

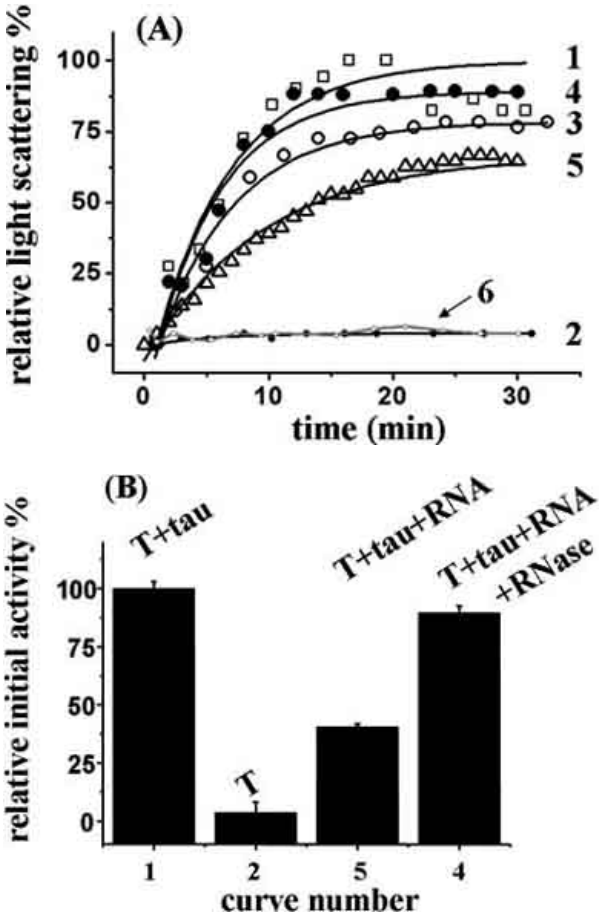

Figure 1. Inhibition of microtubule assembly by RNA.
(A) Protein tau40 was incubated with yeast total RNA at mass ratios 2:1 (curve 3) and 1:1 (curve 5) at 37 °C for 10 min and then was added to tubulin (final concentration 2 μM) followed by the measurement of the light scattering at 350 nm. RNase A (0.1 μg/ml) was added to the mixture of tau40-RNA that had been incubated at 37 °C for 60 min. And then, aliquots were taken for the assembly assays (curve 4). Tubulin alone (curve 2) or protein tau mixed with RNA (curve 6) was used as control. The intensity of light scattering of tau mixed with tubulin (curve 1) at 20 min was indicated as 100%. (B) The relative initial activities of protein tau at different conditions. T represents tubulin.

Similarly, the activity of protein tau in the promotion of actin polymerization or bundling F-actin could be also reactivated by addition of RNase A [our unpublished data]. This suggests that the inactivation of protein tau induced by RNA is reversible under this condition.

To investigate whether protein tau is discriminatively associated with rRNA, tRNA or mRNA, we incubated three kinds of RNA with protein tau for gel mobility shifting assays. As illustrated in (Fig. **2A**), RNA-tau40 complexes become more retarded in agarose gel as the protein concentration increases. The total RNA contains 80% rRNA, indicating that protein tau is bound to rRNA. As shown in (Fig. **2B**) and (**3C**), tau proteins show a strong effect on the shifting of tRNA (lane 2, Fig. **2B**)) and mRNA (lane 2, Fig. **2C**)). BSA as a negative control has no effect on the shifting of mRNA or tRNA (lane 3, Fig. **2B**) and **2C**)). Histone H2B as a positive control shows a markedly effect on the shifting of mRNA (lane 4, Fig. **2C**)), but not tRNA (lane 4, Fig. **2B**)). As mentioned above, protein tau is capable of binding to rRNA, tRNA and mRNA. To prevent the degradation of RNA during the experiments, we added ribonuclease inhibitor (RNasin, 0.5 U/μl) to the reaction mixture. Fortunately, in the experiments, RNasin did not interfere with the binding of protein tau to the yeast total RNA (data not shown).

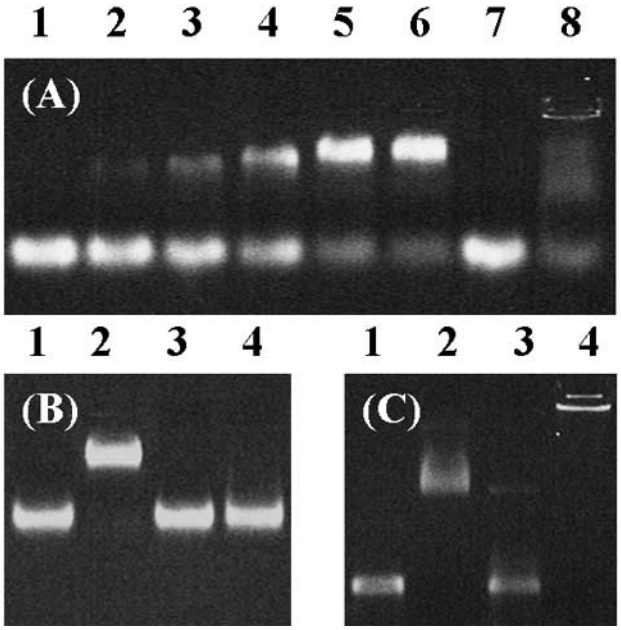

Figure 2. Gel retardation of RNA in the presence of protein tau.
(A) Binding of tau40 to yeast total RNA. RNA (2.5 μg) was incubated in the absence of protein tau40 (lane 1), with increasing concentrations of protein tau40 (from lane 2 to 6), or in the presence of BSA (lane 7, mass ratio 1/1) or histone H2B (lane 8) at 37 °C for 10 min. The mass ratio between RNA and tau40 (from lane 2 to 6) was 5/1, 2/1, 1/1, 1/2, and 1/5, respectively. And then, the mixtures were run on agarose gels. (B and C) Binding of tau40 to tRNA or mRNA. tRNA (2.5 μg) or mRNA (1 μg) was incubated in the absence of tau40 (lane 1) or in the presence of tau40 (mass ratio 1/1, lane 2), BSA (mass ratio 1/1, lane 3), or histone H2B (mass ratio 2/1, lane 4) at 37 °C for 10 min.

Binding of Protein Tau to Both Single and Double Stranded RNA

RNA forms some secondary structures such as hair-pin and double-helical stem, by base pairing between complementary segments of an RNA molecule. In our experiments, an ssRNA ($PolyA_{30}$) and a dsRNA ($PolyA_{15}U_{15}$) have been synthesized to detect whether protein tau is discriminatively bound to single or double stranded RNA. As (Fig. **3**) illustrates, retarded bands of $PolyA_{30}$ and $PolyA_{15}U_{15}$ in the presence of tau23 or tau40 could be detected in EMSA. The shifting of the bands of protein tau40 incubated with $PolyA_{15}U_{15}$ or $PolyA_{30}$ is markedly retarded on the gels. Similar results will be obtained when tau23 is mixed with $PolyA_{15}U_{15}$ or $PolyA_{30}$. Although $PolyA_{15}U_{15}$ forms a double-helical stem while $PolyA_{30}$ is a single strand molecule, the discriminative binding of protein tau to these two kinds of polynucleotide could not be obviously detected. This also implies that protein tau is bound to RNA without nucleotide sequence specificity.

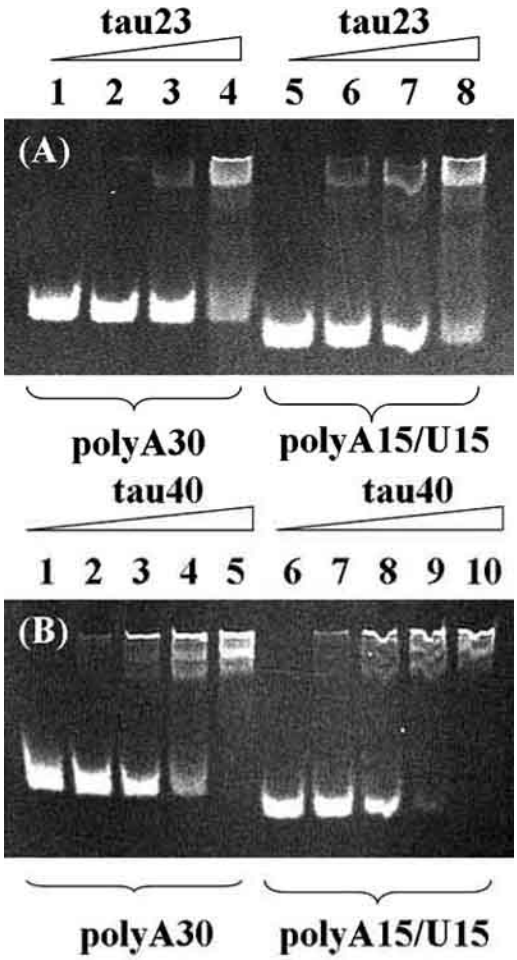

Figure 3. Binding of protein tau23 or tau40 to small RNA.
Synthesized RNA $polyA_{15}U_{15}$ (or $polyA_{30}$) was incubated with protein tau23 or tau40 at 37 °C for 10 min. And then, the mixtures were run on 8% PAGE. (A) The mass ratios of RNA and tau23 in lanes from 2 to 4 and from 6 to 8 are 4:1, 2:1 and 1:1, respectively. Lane 1 and lane 5 were loaded on RNA alone. (B) The mass ratios of RNA and tau40 from lane 2 to 5 and lane 7 to 10 are 4:1, 2:1, 1:1 and 1:2, respectively. Lane 1 and lane 6 were loaded on RNA alone.

Tau Proteins Binding to RNA Observed by Atomic Force Microscopy

RNAs alone such as mRNA or tRNA could not be absorbed onto a mica surface (data not shown). RNA molecule incubated with protein tau, however, can be absorbed onto the mica surface and observed under AFM (Fig. **4**)). Unlike the beads-on-string structure of DNA-tau23 or tau40 complex [15], the complex of protein tau and RNA shows a straw-rope like structure. These results illustrate that protein tau has a high affinity for RNA binding.

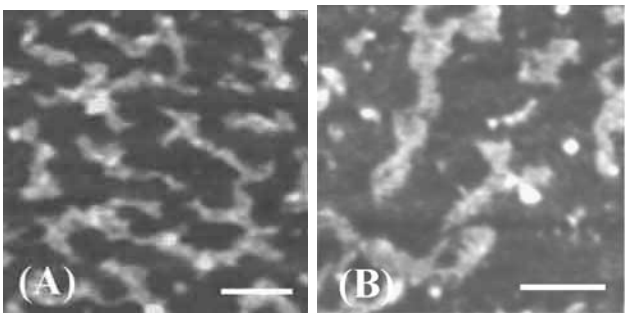

Figure 4. Protein tau binding to RNA observed by AFM.
Protein tau40 was incubated with RNA at 37 °C for 15 min (see Materials and methods section). Samples were diluted 5-10 times and then observed by AFM. Bar is 100 nm in panel A (protein tau mixed with tRNA) and 50 nm in panel B (protein tau mixed with mRNA).

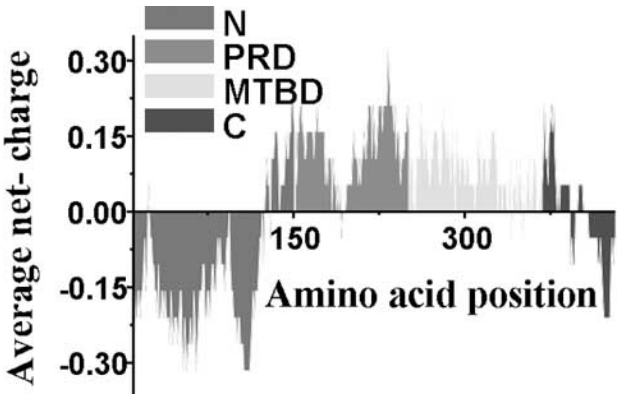

Figure 5. Analysis of charge distribution of protein tau40.
Arg and Lys are positively charged and Asp and Glu are negatively charged. For calculation, these four amino acid residues were considered as the charged components. On the basis of the method by Kyte and Doolittle, a window of 19 amino acid residues is used as a group to estimate the average charge distribution of protein tau40.

Effects of NaCl and pH on Protein Tau Binding to RNA

RNA is negatively charged and protein tau is positively charged in solution. Thus NaCl was used to test whether the association of tau with RNA is due to electrostatic interaction. As shown in (Fig. **6A**), the retardation of RNA is observably reduced as NaCl concentration increases. The reduction is more obvious when the salt concentration is over 0.5 M. That is to say, NaCl (> 0.5 M) decreases the associa-

tion of protein tau with RNA. Furthermore, the retardation of RNA is also reduced under acidic conditions (pH < 5) as shown in (Fig. **6C**). In addition, boiling the complexes of tau and RNA for different time intervals (min) cannot affect on the shifting of the complexes on the gels (Fig. **6B**)). Here, we can conclude that the binding of tau to RNA is due to electrostatic interaction.

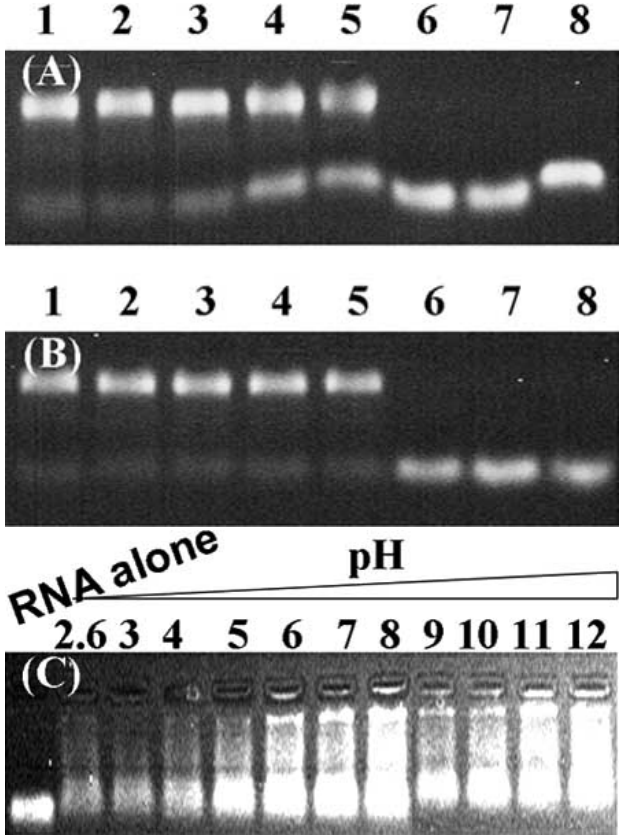

Figure 6. Effects of NaCl, temperature or pH on protein tau binding to RNA.

Yeast total RNA (2.5 μg) was incubated with protein tau40 (mass ratio 1/1) at different concentrations of NaCl, at different pH values or in different boiling time. Samples in the absence of protein tau40 were used as control (last three lanes in panel A and B). Lanes from left to right represent as follows: Samples were (A) mixed with various concentrations of NaCl (0, 0.1, 0.3, 0.5, 1, 0.3, 0.5 or 1 M) at 37 °C for 10 min; (B) boiled at 100 °C for 0, 2, 5, 10, 20, 0, 5 or 10 min; (C) incubated at pH values from 2.6 to 12 at 37 °C for 10 min.

The PRD and the MTBD Acting as RNA Binding Domains

To investigate which domain of protein tau involved in binding to RNA, tau mutants were constructed and expressed in *E. coli* BL21(DE3) strain. And then, they were purified from Ni-NTA column and detected by agarose gels (Fig. **7**). The bands were obviously detected for mutant τMTBD (Fig. **7A**), suggesting that the MTBD is associated with nucleic acids. At the same time, the tau mutant with the MTBD deleted (ΔMTBD) is also associated with nucleic acids. Note that no signal can be detected for τN or τC under the same conditions. Thus, the PRD is another domain involved in binding to nucleic acids. The last band in (Fig. **7A**) for τPro supports this suggestion. To investigate whether the bound nucleic acids are RNA, DNase I or RNase A has been added to the purified proteins before loaded on the gels. As shown in (Fig. **7B**), the bands on the gel can be still observed in the presence of DNase I. This indicates that the associated nucleic acids are mainly RNA molecules. The disappearance of all the bands in the presence of RNase A on the gel confirms that RNA molecules are the bound nucleic acids (Fig. **7C**). These results demonstrate that the proline-rich domain and the microtubule binding domain are definitely acting as RNA binding domains.

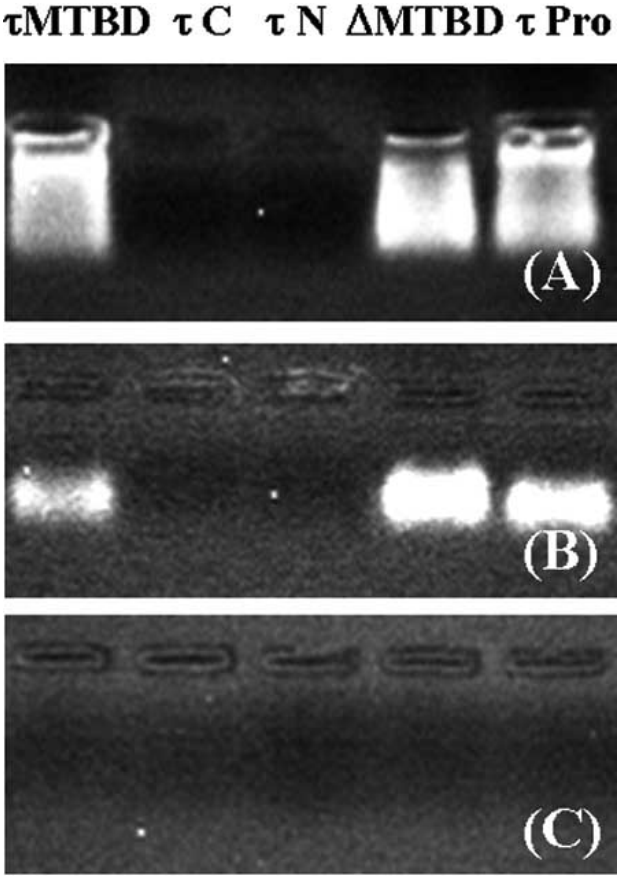

Figure 7. RNA associated with tau-mutants during the purification.

Mutants of tau were purified through Ni-NTA column. Aliquots were run on 1% agarose gels. The purified tau mutants from Ni-NTA column were in the absence of nuclease (A) or in the presence of DNase I (B) or RNase A (C).

Co Localization of Protein Tau with RNAs in Cells

To investigate whether protein tau associated with RNAs, SH-SY5Y cells were stained with RNASelect green fluorescent cell stain in the presence of monoclonal antibody tau-1. As illustrates in (Fig. **8A**), the RNA fluorescent signals are distributed in both cytoplasm and nucleoplasm. The signals, however, are concentrated in nuclei, probably on the nucleoli. As shown in (Fig. **8B**), the immunofluorescence signals of protein tau are also distributed in both cytoplasm and nucleoplasm. Some signals of RNASelect green fluorescence and TRTIC-labeled secondary antibody overlap each other, especially in nuclei (Fig. **8C**). This suggests that a part of protein tau and RNAs are co-localized in the cells.

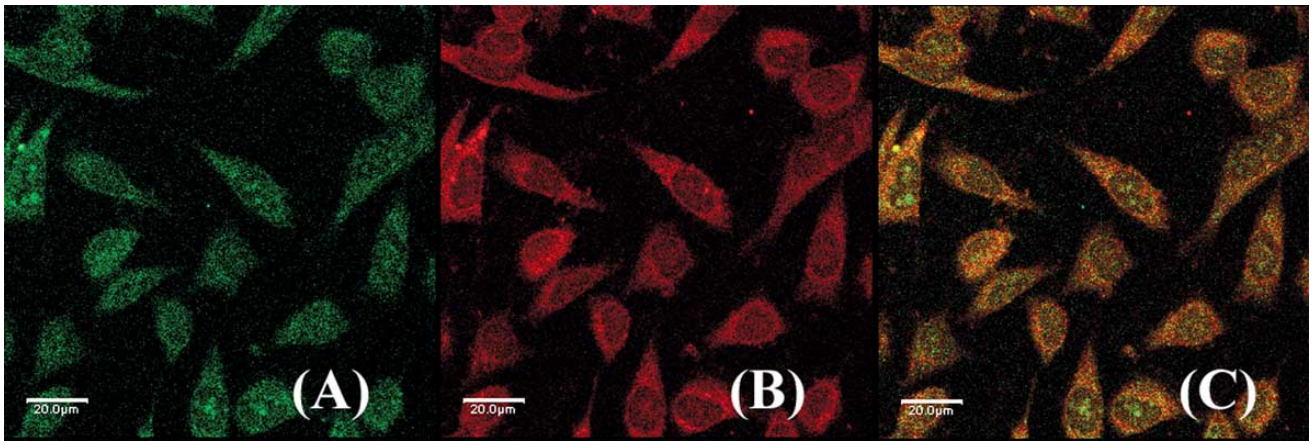

Figure 8. Analysis of the association of cellular tau proteins with RNA.
For co-localization studies of RNA and protein tau in SH-SY5Y cells, the fluorescently labeled cells were visualized with an Olympus confocal microscope using FITC and TRTIC filter sets. SH-SY5Y Cells were stained with SYTO RNASelect green-fluorescent cell stain (A, green). Then they were incubated with tau-1 antibody and TRTIC-labeled secondary antibody (B, red). Yellow color in (C) indicates the overlay of RNA and protein tau.

DISCUSSION

Neuronal tau promotes microtubule assembly and stabilizes microtubules [1-3]. Tubulin, a polyanion, assembles with the help of protein tau or other polycations [17]. Bryan *et al.* [6] found that a tau-like factor is associated with RNA during it purification from sea urchins, Chinese hamster ovary cells and brain. They proposed that spontaneous assembly of microtubules in nonneural cell extracts is blocked because the endogenous factors are complexed with RNA. Results in this work further confirm that RNA inhibits the microtubule assembly-promoting activity of protein tau. Our unpublished data also demonstrated that RNA inhibits the activity of protein tau in the promotion of actin polymerization or bundling F-actin. Experiments *in vitro* suggest that RNA disturbs the interaction of protein tau with other cytoskeleton proteins including actin and tubulin.

Furthermore, protein tau binds nonspecifically to tRNA, rRNA and mRNA. The association of protein tau with RNA is through electrostatic interaction. This viewpoint is based on these observations: (1) Both the proline-rich domain and the microtubule binding domain contain positive net-charge (Fig. **5**) and RNA is a polyanion; (2) Protein tau indiscriminately binds to both double-stranded and single-stranded RNA which are composed of negative-charged phosphate; (3) Both acidic N-terminal and C-terminal region (Fig. **5**) have not been observed to associate with RNA, and (4) the interaction of tau with RNA is markedly interfered in the presence of NaCl (> 0.5 M) or at the acidic solutions (pH < 5). Similarly, the nonspecific electrostatic interaction has been also found in the co-bundling of tau to induce different filament types such as actin and microtubules [18]. These investigations demonstrated that nonspecific electrostatic interactions may dominate the interaction of protein tau with RNA or other cytoskeleton proteins including actin and tublin.

On the other hand, RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments [7]. Ginsberg *et al.* [9] found that RNA is sequestered in the neurofibrillary lesions of Alzheimer's disease. Kampers *et al.* [7] have reported an interesting result on the interaction between RNA and tau whose MTBD is the most important region involved in the tau assembly. According to their observations, the mutant (K23) could not be found to form PHFs in the presence of RNA. In this work, however, the PRD has been definitely detected to associate with both single and double stranded RNA. This different result may be due to that Kampers' observations focus on the formation of RNA-induced PHFs. In addition, the primary structure of mutant τPRD is different from the K23 mutant that contains the PRD. Last year, by using NMR secondary chemical shifts, Mukrasch *et al.* [19] detected the residual β-structure for 8–10 residues at the beginning of repeats R2–R4 of protein tau. These regions correspond to sequence motifs known to form the core of the cross-β-structure of tau-paired helical filaments. Thus, that the assembly of PHFs from mutant K23 by RNA could not be detected may be due to the lack of motives of the PRD to form a core of cross-β-structure though the PRD has been definitely detected to associate with RNA in our experiments.

Interestingly, one member of the microtubule-associated proteins, microtubule- associated protein 1 A (MAP 1A) has been found to mediate the general attachment of mRNA to the cytoskeleton through the formation of the ribonucleoprotein complex [20]. Our results in SH-SY5Y cells show that protein tau and RNA are co-localized in both nuclei and cytoplasm, indicating that RNA may have chances to functionally bind to protein tau in cells or induce protein tau to form PHFs. However, whether protein tau interacts with RNA *in vivo* needs further investigation.

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(STZ98-2-07) and Equipment Foundation of the Chinese Academy of Sciences.

ABBREVIATIONS

PRD	=	Proline-rich domain
MTBD	=	Microtubule binding domain
PHFs	=	Paired helical filaments
AFM	=	Atomic force microscopy
ΔMTBD	=	tau23 mutants with the MTBD deletion
τN	=	Truncated tau23, which is tau N terminus
τPro	=	Truncated tau23, which is the proline-rich domain of tau
τMTBD	=	Truncated tau23, which is the microtubule binding domain of tau
τC	=	Truncated tau23, which is tau C terminus.

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