TRF2 is in neuroglial cytoplasm and induces neurite-like processes

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Abstract TRF2 is a ubiquitous protein that protects telomeres in the nucleus. We found that TRF2 was present at the peripheral nerve axons and the brain neuroglial cell processes extensively. It was in the cytoplasmic membrane as well as nuclear fractions, but not in the soluble cytoplasmic fraction of SH-SY5Y neuroblastoma cells. *TRF2* was up-regulated in P19 embryonal carcinoma cells at the early stage of induced neural differentiation with retinoic acid treatment. Upon transfection, TRF2-expressing COS cells often produced neurite-like long cytoplasmic processes. TRF2 is a component of neuroglial cells and appears to be involved in the cytoplasmic process formation that is necessary for neural differentiation.

Key words: TRF2; Neural differentiation; Glia; Axon; Neurite

1. Introduction

TRF2 (TTAGGG repeat binding factor 2) is a nuclear protein that coats the length of all human telomeres at all stages of the cell cycle [1,2]. It protects telomeres from end-to-end fusion [3] by forming large duplex loops at the end [4]. It delays replicative senescence of cultured cells by protecting critically short telomeres from fusion [5].

TRF2 inhibition leads to immediate induction of apoptosis suggesting that TRF2-depleted telomeres are perceived as if they represent sites of DNA damage [6]. TRF2 binds with several telomere-regulating proteins such as hRap1 [7], Mre11 complex [8], Ku [9], and Werner and Bloom syndrome helicases [10], suggesting that it protects and maintains the telomere function in collaboration with other proteins. The down-regulation of TRF2 has been implicated in malignancies [11,12].

We have screened the expression of TRF2 in various tissues using affinity-purified anti-TRF2 antibody [9]. Upon immunohistochemical staining, we unexpectedly found that TRF2 was present not only in the nuclei but also at the axon of peripheral nerves and brain tissue. Here, we show that TRF2 is a neuroglial cell component and induces neurite-like cytoplasmic process formation. Our results suggest that TRF2 has a novel function of neurite formation that is not related to the telomere-protecting function.

2. Materials and methods

2.1. Immunohistochemistry

Paraffin-embedded and/or fresh human tissue samples from various organs were obtained from autopsy and surgical pathology files at Asan Medical Center, Seoul, Korea, with informed written consent. For the immunohistochemical staining, sections of 4 μm thickness were prepared from paraffin blocks. Microwave antigen retrieval was applied in 0.01 M sodium citrate buffer (pH 6.0). The affinity-purified anti-TRF2 antibody was produced against recombinant TRF2 expressed in *Escherichia coli* [9]. For negative control, antigen pre-absorbed antibody was applied. Anti-TuJ1 (Covance, Richmond, CA, USA), anti-NeuN (Chemicon, Temecula, CA, USA), anti-synaptophysin (DakoCytomation, Carpinteria, CA, USA), and anti-neurofilament (DakoCytomation) antibodies were applied for 2 h at room temperature similarly. After washing, immunostaining was done using the avidin-biotinylated horseradish peroxidase complex method, and was developed by immersing slides in diaminobenzidine as chromogen.

2.2. Tissue culture and induced neural differentiation

SH-SY5Y [13] and COS cells were grown at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml). P19 cells obtained from the American Type Culture Collection (Rockville, MD, USA) [14] were cultured in α-minimal essential medium (Invitrogen) supplemented with 7.5% fetal bovine serum and 2.5% fetal calf serum (Invitrogen). To induce neuronal differentiation, SH-SY5Y cells were incubated in complete medium plus 1 μM all-*trans* retinoic acid (Sigma, St. Louis, MO, USA) for 5 days [15].

P19 cells were grown in 100 mm bacteriological grade Petri dishes. To induce neural differentiation, all-*trans* retinoic acid was added to the medium to a final concentration of 0.5 μM [16]. After 4 days, the cell aggregates were trypsinized, transferred into tissue culture dishes and cultured for an additional 5 days in the same medium without retinoic acid. Cells were harvested every 24 h and analyzed by Western blot for 5 days.

2.3. Cell fractionation

Cell fractionation experiments were repeated twice independently. SH-SY5Y cells were washed with cold phosphate-buffered saline (PBS), scraped from the plates and allowed to swell in 0.5 ml of lysis buffer (50 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 0.1% (v/v) Triton X-100) on ice for 10 min. Then, cells were homogenized with 10 gentle strokes of a glass-to-glass Dounce homogenizer. The homogenates were first centrifuged (at 600×*g* and 4°C for 20 min) to obtain a crude nuclear pellet and a soluble fraction. The purity of the nuclear preparations was moni-

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Abbreviations: TRF2, TTAGGG repeat binding factor 2

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tored routinely by phase contrast microscopy after staining with methylene blue. The soluble fraction was further centrifuged (at 100 000×*g* and 4°C for 30 min) to yield a cytoplasmic soluble supernatant and a plasma membrane pellet. The crude nuclear pellet was washed with the lysis buffer, layered over 45% (w/v) sucrose, centrifuged (at 1660×*g* and 4°C for 30 min), resuspended and sonicated in the lysis buffer containing 1% (v/v) Triton X-100. Then, the homogenate was centrifuged (at 14 000×*g* and 4°C for 30 min) to yield soluble and insoluble nuclear fractions.

2.4. Western blot

SH-SY5Y cell fractions, retinoic acid-treated P19 cells, or tissue samples were solubilized in the lysis buffer. Protein concentrations were measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), and the same amount of total proteins was loaded in each lane. After 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the separated peptides were transferred to nitrocellulose paper. Affinity-purified anti-TRF2 antibody and anti-TuJ1 antibodies were used as primary antibodies. For the control, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody was used similarly. Immune complexes were visualized using the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK). The intensities of bands were determined densitometrically using a Multi-Image Analysis System (Bio-Rad Laboratories). Data were expressed as arbitrary densitometric values normalized against β-actin in fold increase of control.

2.5. Transfection and immunofluorescence microscopy

COS cells grown on coverslips were transfected using pcDNA3-TRF2/T7·Tag or pcDNA3-TRF2$^{\Delta B\Delta M}$/T7·Tag, a mutant deleted in the N-terminus and the myb-like C-terminus [9]. Cells were transfected with 3 μg of plasmid DNA/60 mm dish using LipofectAMINE PLUS™ reagent (Invitrogen). Three experiments were performed independently. For a negative control, cells were transfected with the vector alone. At 36 h after transfection, cells were fixed in cold methanol at −20°C, and processed for immunofluorescence microscopy. Affinity-purified anti-TRF2 antibody and/or monoclonal anti-T7·tag antibody (Novagen, Madison, WI, USA) were applied as primary antibodies. As controls, normal rabbit and/or mouse sera were used. Rhodamine-conjugated anti-rabbit IgG secondary antibody and/or fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody (Zymed, South San Francisco, CA, USA) were applied as second antibodies for 30 min. After washing with PBS three times, nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma) and mounted. Cells were viewed using an Olympus Vanox-S fluorescence microscope.

3. Results and discussion

3.1. TRF2 is present at the peripheral nerve axons and brain neuroglial cell processes

To understand the *TRF2* function further, we surveyed the expression in human tissues by immunohistochemistry using affinity-purified rabbit antisera [9]. In every organ, most cells were immunostained in the nuclei, although the staining intensity varied considerably. Unexpectedly, we found that peripheral nerve axons were strongly immunostained regardless of the size or location (Fig. 1A). Similarly, the brain was also extensively immunostained while antigen pre-absorbed control antibodies did not show any staining. All neurons had intense TRF2 immunostaining throughout the soma and processes displaying the complex neural network of the brain (Fig. 1B). There was also a finely fibrillar staining in the white matter, where no neuron expressing synaptophysin was present, indicating that TRF2 was also present in the glial processes (Fig. 1C,D). The distribution of TRF2 in the central nervous system was reminiscent of TuJ1 (neuron-specific class III β-tubulin) [17] except that nuclei were negative on TuJ1 immunostaining (Fig. 1E). NeuN was expressed in the neuronal nuclei and soma similarly [18], but it was not expressed in glial cells (Fig. 1F). Neuroblastomas and gliomas, which are malignant neural and glial tumors, also strongly expressed TRF2 at the neural and glial processes, respectively (Fig. 1G,H).

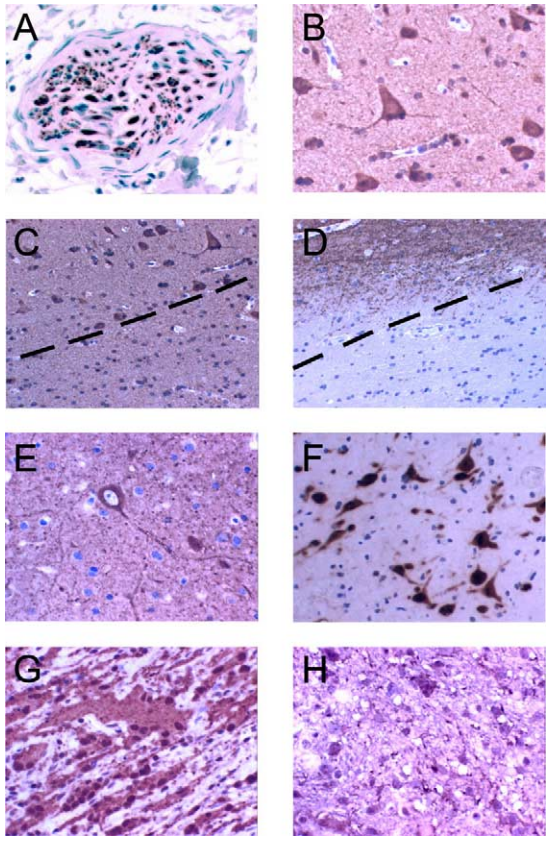

Fig. 1. TRF2 is a component of the peripheral and central nervous system. A: Immunohistochemical staining of peripheral nerve showing intense staining of TRF2 at the axons (brown color). B: Brain neurons also express TRF2 in the soma and neurites extensively. C: TRF2-positive network in both gray matter (upper part) and white matter (lower part) of the brain. D: In the step section of C, the boundary of gray matter is shown by expression of synaptophysin, a neural marker. E: TuJ1 immunostaining of neuronal soma and processes, but nuclei are negative. F: NeuN immunostaining in both neuronal nuclei and cytoplasm. G: Neoplastic neural cells in neuroblastoma also express TRF2 in the soma, neurites and nuclei. H: Neoplastic glial cells in a glioma also express TRF2 in the delicate glial processes. (A,B,E–H: ×100; C,D: ×50). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As reported previously, TRF2 was detected as two bands of 65 and 69 kDa upon Western blotting [1,2]. The expression of TRF2 varied considerably in different organs: it was much higher in the brain than in organs such as thymus, kidney, lung and liver (Fig. 2A). β-Actin, which was taken as a control, was also highly expressed in the brain. From the densitometric analysis of immunoblots, the TRF2 expression normalized against β-actin was still higher in the brain than in other organs (Fig. 2B).

3.2. TRF2 is in the nuclear and cytoplasmic membrane fractions but not in the soluble cytoplasmic fraction of neuroblastoma cells

We then analyzed in which cellular fraction TRF2 was present. SH-SY5Y neuroblastoma cells were treated with

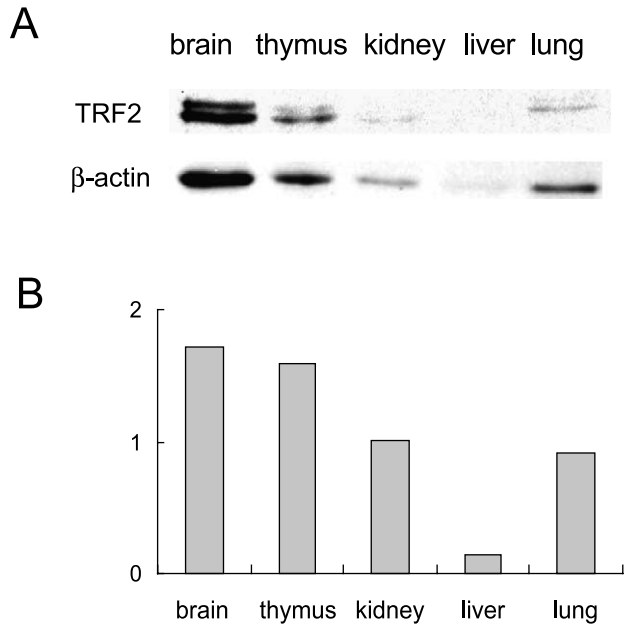

Fig. 2. Western blot for TRF2 in human tissues. A: Upon loading of 30 μg of total proteins in each lane, the TRF2 expression is much higher in the brain than in other tissues. The control β-actin is also highly expressed in the brain. B: From the densitometric analysis of immunoblots, the TRF2 expression normalized against β-actin was still higher in the brain than in other organs.

1 μM retinoic acid to induce neural differentiation [15]. Cells were harvested after 5 days when more than 90% of cells showed TuJ1-positive neurites, and cellular fractions were prepared as described in Section 2. Upon 10% polyacrylamide gel electrophoresis and immunoblotting, TRF2 was detected in the cytoplasmic membrane fraction as well as in the soluble and insoluble nuclear fractions, but not in the soluble cytoplasmic fraction (Fig. 3). TRF2 consisted of two bands with similar proportions in each fraction. From the densitometric analysis, it was estimated that the amount of TRF2 in the cytoplasmic membrane fraction was about 45% of the total TRF2 in the differentiated SH-SY5Y cells.

3.3. *TRF2 is up-regulated at the early stage of induced neural differentiation of P19 cells*

The abundance of TRF2 in the cytoplasmic membrane fraction suggested a particular function in the nervous system, distinct from the telomere regulation. We checked the expression of TRF2 during the experimental neural differentiation. For the experiment, we used P19 embryonal carcinoma cells instead of SH-SY5Y cells, because the former did not show any visible 'leakage' of neurite formation in the culture conditions without retinoic acid. After the exposure to 0.5 μM retinoic acid for 4 days, P19 cells were replated in tissue culture flasks. On immunoblotting, TuJ1 began to be detected 48 h post treatment and increased exponentially afterwards, indicating the induction and ongoing neural differentiation (Fig. 4A,B). TRF2 also increased after the retinoic acid treatment, but in a distinct pattern from TuJ1: the TRF2 expression normalized against β-actin increased up to 2.19 times of the basal level on the third day, and began to decrease afterwards (Fig. 4A,C). The expression of β-actin did not change significantly for 5 days. The TRF2 up-regulation before TuJ1 induction suggested that TRF2 might have a direct role in the induction of neurites at the early stage of neural differentiation.

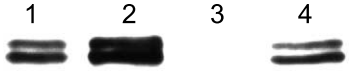

Fig. 3. TRF2 is present in the nuclear and cytoplasmic membrane fractions but not in the cytoplasmic soluble fraction of neuroblastoma cells. SH-SY5Y cells were induced to undergo neural differentiation using 1 μM retinoic acid, and cellular fractions were prepared as described. TRF2 was detected as two bands of 65 and 69 kDa with similar proportions among the fractions. Total protein 20 μg was loaded in each lane: 1, soluble nuclear fraction; 2, insoluble nuclear fraction; 3, soluble cytoplasmic fraction; and 4, cytoplasmic membrane fraction.

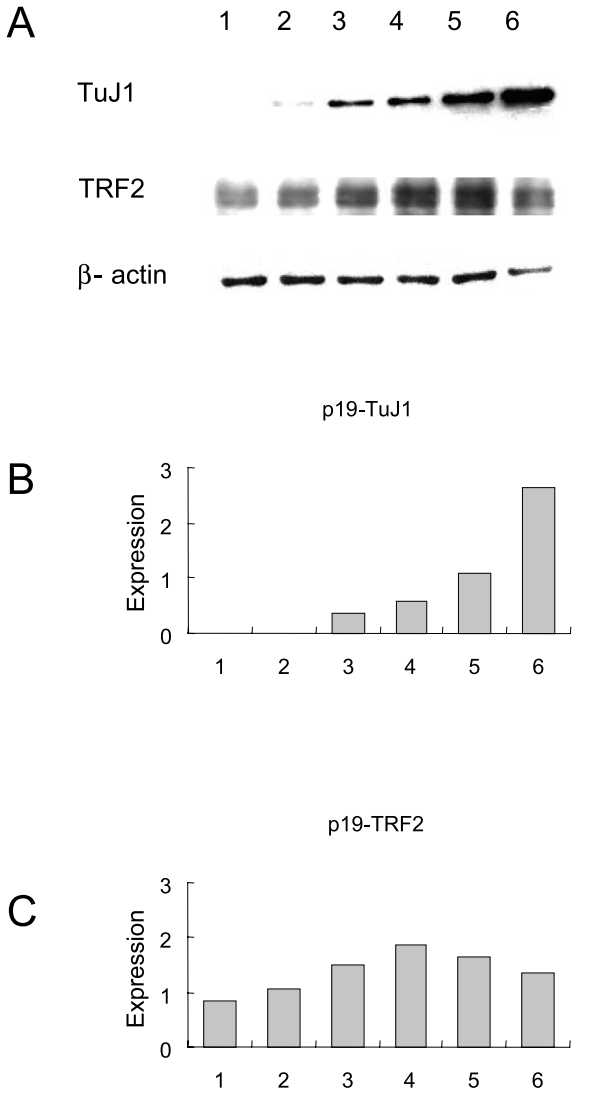

Fig. 4. TRF2 up-regulation at the early stage of induced neural differentiation. A: Immunoblot using anti-TuJ1, TRF2, and β-actin antibodies. P19 embryonal carcinoma cells were treated with 0.5 μM retinoic acid and harvested every 24 h. Lane 1: untreated control, lanes 2–6: days 1–5 after treatment. Total protein 20 μg was loaded in each lane. B: The expression of TuJ1 normalized against β-actin expression as a control. C: The normalized expression of TRF2.

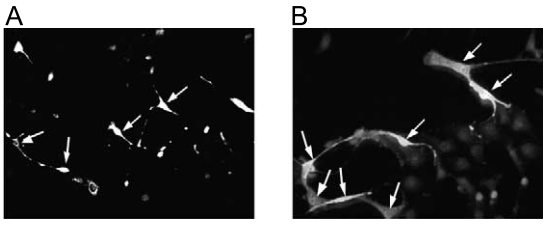

Fig. 5. COS cells transfected with *TRF2* produce neurite-like processes. A,B: Immunofluorescence microscopy of COS cells harvested 36 h after transfection using monoclonal anti-T7·Tag and rabbit anti-TRF2 antibodies, respectively (×50 and ×100). Transfected cells often display neurite-like processes with strong immunofluorescence staining for TRF2. The long and slender processes are often branching and connected to other cells. Nuclei are designated with arrows. Both pcDNA3-*TRF2*/T7·Tag or pcDNA3-*TRF2*$^{\Delta B\Delta M}$/T7·Tag, a mutant deleted in the N-terminus and the myb-like C-terminus, show similar results.

3.4. *TRF2 transfection induces neurite-like processes in COS cells*

We then examined whether *TRF2* could induce neurite-like processes in cultured cells. COS cells were transfected with either pcDNA3-*TRF2*/T7·Tag or pcDNA3-*TRF2*$^{\Delta B\Delta M}$/T7·Tag, a mutant deleted in the N-terminus and the myb-like C-terminus [9]. For negative control, cells were transfected with the vector alone. At 36 h post transfection, cells were processed for immunofluorescence microscopy using anti-TRF2 and/or anti-T7·Tag antibodies. The transfection efficiency varied from 3 to 10% among three repetitions. Transfected COS cells showed strong immunostaining in the nuclei and/or cytoplasm. Among the TRF2-transfected COS cells, about 10–20% had slender cytoplasmic processes, which were not present in the mock transfection. They had two or more processes that were up to 20–30 times of the nuclear length (Fig. 5A,B): the nuclei were small and slender in comparison with other cells. Transfected cells had either bipolar or multipolar processes. The processes were quite reminiscent of neurites: they often showed a beaded appearance, branched and connected to adjacent cell processes. However, they did not express neural markers such as TuJ1, NeuN, neurofilament, or synaptophysin on double immunostaining (data not shown). The neurite-like processes were found on both full-length *TRF2* and *TRF2*$^{\Delta B\Delta M}$ transfection, indicating that neither the N- nor the C-terminus was involved in the neurite-like process formation.

Taken together, TRF2 is present abundantly in the cytoplasmic membrane fraction of neural cells, and appears to induce the neurite-like cytoplasmic processes. Our data suggest that the neurite formation may precede further biochemical steps toward mature neurons. The molecular mechanisms of neural process initiation and growth have not been well elucidated. It has been shown that Rho-GTPases and Cdc42 are involved in the axon initiation and growth [19], and the regulation of actin polymerization through the Arp2/3 complex [20]. Given that *TRF2* induces neurite-like processes in non-neural cells, it is likely that TRF2 functions upstream of those pathways in the complex molecular mechanism of neural differentiation.

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