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Morphological characterization of mammalian Timeless in the mouse brain development

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ABSTRACT

Timeless was originally identified in Drosophila as an essential component of circadian cycle regulation. In mammals, the ortholog of Timeless (Tim) has also implicated in cell cycle control and embryonic development. In this study, we generated a specific antibody against Tim, and carried out expression and localization analyses of Tim during mouse brain development. In Western blotting, Tim was detected throughout the developmental stage. In immunohistochemical analyses, Tim was detected strongly in neurons in the ventricular zone/subventricular zone and moderately in cortical neurons during corticogenesis. In adult mouse brain, Tim was observed moderately in cortical neurons. Notably, Tim was enriched in the nucleus of cortical neurons from embryonic to early postnatal stages while it was distributed in the cytoplasm in the adult stage. Similar distribution change from nucleus to cytoplasm was observed in the hippocampal neurons between P0 and P30. In situ hybridization revealed that the tissue expression profile of Tim-mRNA was similar to that of the protein. In differentiated primary cultured mouse hippocampal neurons, Tim was detected in cell body, axon and dendrites. The obtained results suggest that Tim is expressed in neuronal tissues in a spatiotemporally regulated manner and involved in developmental stage-specific neuronal functions.

1. Introduction

Timeless was originally identified in Drosophila as an essential component of the circadian rhythm regulation, and is conserved in all eukaryotes (Reppert, 1998). However, no consensus has been established regarding whether mammalian Timeless (Tim) indeed plays an essential role in the circadian clock machinery (Gotter, 2006). While the Tim gene was shown to be expressed in the suprachiasmatic nuclei (SCN), the brain circadian clock center, most reports detected little or low circadian oscillations in Tim mRNA or protein level in the SCN (Koike et al., 1998; Sangoram et al., 1998; Takumi et al., 1999; Zylka et al., 1998). Although Tim function specific in the circadian clock mechanism remains to be controversial, Tim has been implicated in the cell cycle control (McFarlane et al., 2010). In addition, Tim was reported to link the circadian rhythm to cell cycle, and may have additional function in DNA damage control (Unsal-Kaçmaz et al., 2005; Gotter et al., 2007). On the other hand, genetic studies have shown that Tim is essential for early embryonic development, as homozygous knockout produces early embryonic lethality in mice (Gotter et al., 2000). In this context, Tim has been reported to be crucial in the developmental process of lung and kidney (Li et al., 2000; Xiao et al., 2003). Based on these results, Tim is possible to have an important role during the development of mammalian central nervous system (CNS). However, physiological role of Tim in CNS remains to be elucidated not only in developmental but also in adult stages. In the present study, we prepared a specific antibody against Tim and performed some morphological, cell biological and biochemical analyses of Tim by focusing mouse brain development.

2. Materials and methods

2.1. Plasmids

Mouse Tim cDNA was a kind gift from Dr. Smithgall (University of Pittsburgh) (Genbank accession no. AF126480), and

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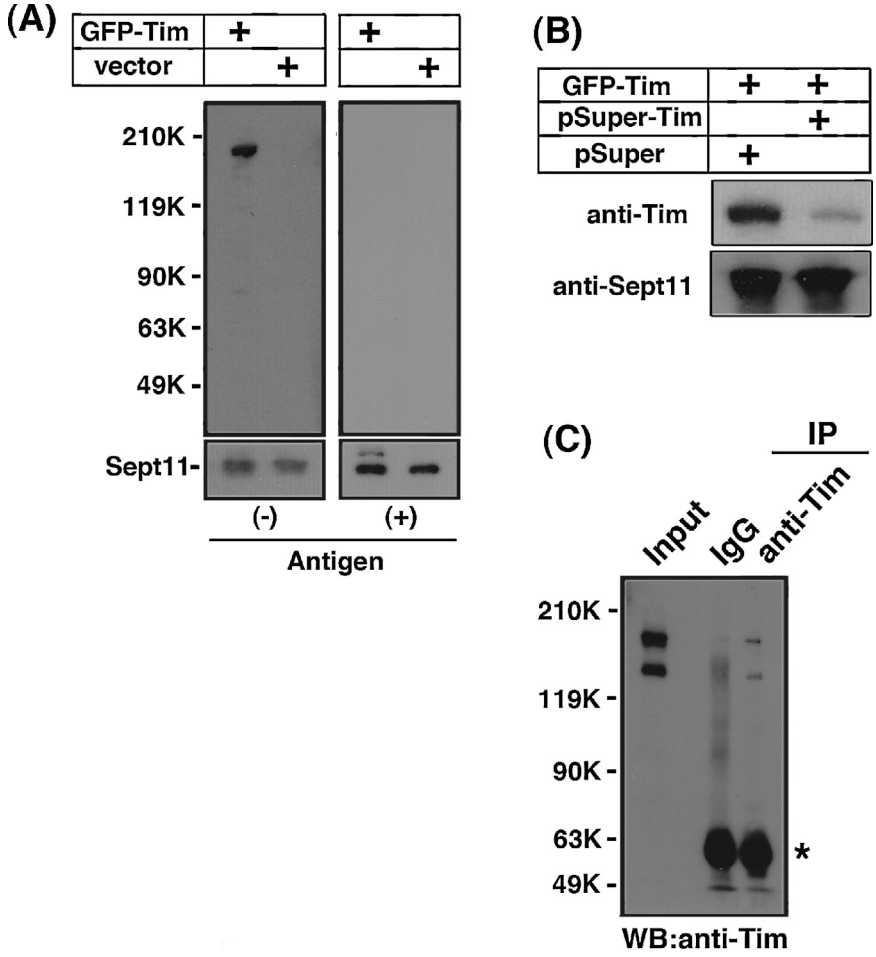

Fig. 1. Production of an affinity-purified antibody for mammalian Timeless, anti-Tim. (A) Lysates (20 μg of protein per lane) from COS7 cells transiently expressing GFP-Tim or control vector were subjected to SDS-PAGE (10% gel) followed by western blotting with anti-Tim (upper left panel) or the antibody preabsorbed with the antigen (upper right panel). The blots were reprobed with anti-Sept11 antibody for the loading control (lower panels). Molecular size markers were shown at left. (B) COS7 cells were transfected with pSuper vector or pSuper-Tim together with pCAG-GFP-Tim. After 48 h, cells were collected and lysates (20 μg of protein) were subjected to Western blotting using anti-Tim. The blot was reprobed with anti-Sept11. (C) Immunoprecipitation of Tim by anti-Tim. Lysates (100 μg of protein) from COS7 cells expressing GFP-Tim were immunoprecipitated with anti-Tim. In the control experiment, rabbit IgG was used. The precipitated materials (20%) and the input (5%) were subjected to Western blotting with anti-Tim. IgG heavy chain was marked (*).

constructed into pCAG-EGFP vector (Addgene Inc., Cambridge, MA). For RNAi experiments, the mouse Tim target sequence (GAAGAAACGGTTTCAGATT, 3555–3573) was inserted into pSuper-puro vector (OligoEngine, Seattle, WA), which was named as pSuper-Tim. Numbers indicate the positions from translational start sites. All constructs were verified by DNA sequencing.

2.2. Preparation of anti-Tim antibody

Using glutathione S-transferase (GST)-fused mouse Tim C-terminal fragment (aa 981–1198) expressed in *Esherichia coli* as an antigen, a rabbit polyclonal antibody (anti-Tim) was generated and affinity-purified on a column to which the antigen had been conjugated.

2.3. Antibodies

The following antibodies were used; monoclonal mouse anti-synaptophysin (Progen Biotechnik, Heidelberg, Germany), anti-glial fibrillary acidic protein (GFAP) (Santa Cruz Biotech, Santa Cruz, CA), anti-Tau-1 (Chemicon International, Temecula, CA), and anti-MAP2 (Sigma–Aldrich, St Louis, MO). Polyclonal rabbit antibody against a cytoskeleton-related protein, Sept11 (anti-Sept11) was prepared as described (Hanai et al., 2004).

2.4. Animals

Timed-pregnant and postnatal ICR mice were from Japan SLC Inc. (Shizuoka, Japan). Anesthesia was carried out as follows. For Western blotting, mice were deeply anesthetized intraperitoneally with pentobarbital (100 mg/kg body weight). For immunohistochemistry, anesthetic containing 1 mg/ml of medetomidine (Nippon Zenyaku Kogyo, Tokyo, Japan), 5 mg/ml of midazoram (Astellas Pharma, Tokyo) and 5 mg/ml of butorphanol (Meiji Seika Kaisha, Tokyo) was injected intraperitoneally at 0.08 ml/10 g body weight (Kirihara et al., 2013). Deeply anesthetized mice were perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brains were post-fixed by immersion in 4% PFA overnight at 4 °C. As for in situ hybridization, pregnant mice were deeply anesthetized with sodium pentobarbitone (Somnopentyl) at 100 mg/kg body weight (Kyoritsu Pharmaceuticals, Tokyo). Embryos were perfused transcardially with 4% PFA in PBS. Postnatal mice were also anesthetized and perfused with 4% PFA as above. All mice were kept at ~37 °C until sacrifice.