Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Neurochemistry International 50 (2007) 251–255
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

The production of antibodies that distinguish rat choline acetyltransferase from its splice variant product of a peripheral type

Shin Kimura, Jean-Pierre Bellier, Akinori Matsuo, Ikuo Tooyama, Hiroshi Kimura *

Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu 520-21, Japan

Received 23 July 2005; received in revised form 9 August 2006; accepted 9 August 2006
Available online 29 September 2006

Abstract

To produce antibodies that permit the immunohistochemical discrimination of choline acetyltransferase of the common type (cChAT) from its splice variant of a peripheral type (pChAT), we immunized rabbits with a cChAT specific recombinant protein encoded by ChAT exons 7 and 8 of the rat cChAT gene. Successful antibody production was proved by Western blotting on rat brain and on HEK293 cells expressing green fluorescent protein (GFP), cChAT-GFP and pChAT-GFP. By immunohistochemistry our antiserum clearly labeled known cholinergic structures in rat brain, but gave no positive staining in the trigeminal ganglion which contained many neurons positive with pChAT antiserum.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Choline acetyltransferase; Immunohistochemistry; Recombinant fusion proteins; Antibody production; Western blotting; Green fluorescent protein

1. Introduction

Antibodies against acetyl-CoA:choline *O*-acetyltransferase (ChAT; E.C. 2.3.1.6.), the synthetic enzyme of acetylcholine (ACh), have been used to label ACh-containing cells by immunohistochemistry (Cozzari and Hartman, 1980; Kimura et al., 1980, 1981; Eckenstein and Thoenen, 1982; Levey et al., 1982; Ichikawa et al., 1987; Houser, 1990) The protein ChAT, being encoded by a successive splicing from exon 2 to exon 15 in the rat, has been found in both the central and peripheral nervous systems (Hahn et al., 1992). In 2000, Tooyama and Kimura identified the presence of a splice variant of ChAT mRNA that lacks a serial sequence corresponding to coding exons from 6 to 9 (Tooyama and Kimura, 2000). Both the variant mRNA and its protein product were found predominantly in the peripheral nervous system by RT-PCR and immunohistochemical analyses. The novel protein was thus termed ChAT of a peripheral type (pChAT), and the well-studied one was ChAT of a common type (cChAT). Prior to the identification of pChAT, various antibodies against cChAT have been produced (Cozzari and Hartman, 1980; Kimura et al., 1980; Eckenstein and Thoenen, 1982; Levey et al., 1982; Ichikawa et al., 1987; Munoz-Maines et al., 1988; Hahn et al., 1992; Ostermann-Latif et al., 1992; Benecke et al., 1993; Poethke et al., 1997). Because both the C- and N-terminal peptide sequences of pChAT are contained in cChAT, it is possible that some of these cChAT antibodies may cross-react with pChAT. To produce a specific antibody against cChAT but not pChAT, it is theoretically promising to choose an antigenic peptide involved only in cChAT. Such a peptide with potential antigenicity may be found in a middle assembly encoded by rat cChAT exon 6–9. Here we report that an antiserum raised against a peptide encoded by exons 7 and 8 is capable of visualizing cells and fibers containing cChAT, but not pChAT, in the rat by immunohistochemistry.

2. Experimental procedure

2.1. Cloning and purification of the antigenic peptide

As shown in Fig. 1, the amino acid sequence which is present in cChAT but not in pChAT is encoded by a contiguous sequence from exon 6 to exon 9 of the rat ChAT gene. To produce a recombinant peptide encoded by exons 7 and 8, the two exons were amplified with PCR using cDNA from the rat striatum as a template and the following primers.

- Upper primer: 5′-GGATCCTTCTTTGTCTTGGATGTTGTCAT-3′.
- Lower primer: 5′-CTGCAGGGACTTGTCATA-3′.

The BamH I site was added to the 5′-terminal region of the upper primer. The Pst 1 site exists in the 3′-terminal of the exon 8, corresponding to the 5′-terminal

* Corresponding author. Fax: +81 775 48 2331.
E-mail address: hkimura@belle.shiga-med.ac.jp (H. Kimura).

0197-0186/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2006.08.011

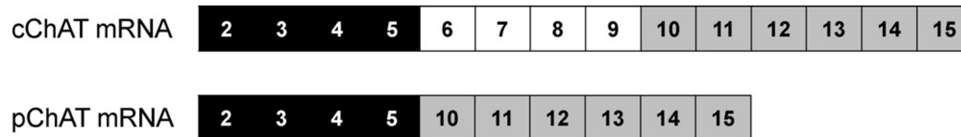

Fig. 1. Two forms of mRNA coding for the common form (cChAT) and alternatively spliced (pChAT) ChAT transcripts. Numerals in boxes indicate protein-coding exons of the genomic DNA coding for cChAT protein (Hahn et al., 1992).

of the lower primer. The RT-PCR product was first cloned into pCR2.1 vector (Invitrogen Japan, Tokyo, Japan), and then replaced into pQE30 vector (QIAexpressionistTM, QIAGEN K.K., Tokyo, Japan) using the restriction enzymes of BamH I and Pst I (Nippon Gene, Tokyo, Japan) and T4 ligase (Takara, Otsu, Japan). The fusion protein consisted of 116 amino acids corresponding to 204–319 of the rat cChAT protein sequence (FFVLDV-VINFRRLSEGDLFTQLRKIVKMASNEDERLPPIGLLTSDGRSEWAKAR-TVLLKDSTNRDSLDMIERCICLVCLDGPGTGELSDTHRALQLLHGGGC-GLNGANRWYDKSLQ: MW = 12,949) encoded by rat cChAT exons 7 and 8, plus 12 amino acids of the N-terminal Tag (MRGSHHHHHHGS) and 5 amino acids of 3′ region (PSLIS). The molecular size of the fusion protein was about 14.8 kDa. The inserted vector was infected to *E. coli* (XL1-blue, Toyobo, Osaka, Japan) and then the bacteria were induced with 1 mM isopropylthio-β-D-galactosidase (IPTG). Amplified *E. coli* were lysed by sonication in extraction buffer containing 500 mM NaCl, 0.05% Tween 20 and 1 mg/ml lysozyme in 20 mM Tris–HCl (pH 7.5).

The lysate was centrifuged, and the supernatant was purified by ammonium sulfate precipitation (30–70%), followed by a chromatography on a column packed with nickel-nitrilotriacetic (Ni-NTA) resin. After washing with 100 mM NaH_2PO_4 containing 8 M urea and 10 mM Tris–HCl buffer (pH 6.3), the 6-His tagged peptide bound to the resin was eluted with 100 mM NaH_2PO_4 containing 8 M urea and 10 mM Tris–HCl buffer (pH 3.0). When examined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with silver staining, a single band with the expected size (14,845 Da) was obtained (Fig. 2, lane C). Our partial N-terminal sequencing, performed on an Applied Biosystems Procise® 492 cLC protein sequencer, indicated that the initial 25 amino acids perfectly matched with those of the peptide encoded by exons 7 and 8 (data not shown).

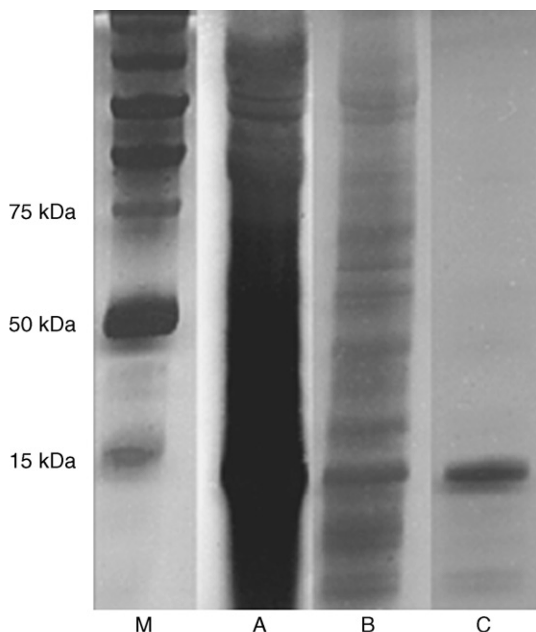

Fig. 2. Silver staining on a SDS-PAGE. The crude protein extract (lane A), the fraction after ammonium sulfate precipitation (lane B), and the purified protein of about 14.8 kDa after the Ni-NTA chromatography (lane C). M indicates a molecular weight marker.

2.2. Immunization

Three white Japanese rabbits (Kiwa Lab., Wakayama, Japan) weighing 2.0–2.5 kg were used. All procedures with animals were designed to minimize the number of animals and their suffering in accordance with the NIH *Guide for Care and Use of Laboratory Animals* (1996) and as approved by the Institutional Animal Care and Use Committee of Shiga University of Medicine. The rabbits received injections of the purified 6-His tagged peptide (14.8 kDa) emulsified with an adjuvant at 2-week intervals. For the first and second injections the adjuvant TiterMax (TiterMax USA, GA) was used, and for later immunizations Freund's incomplete adjuvant (Wako, Osaka, Japan) was used. Each rabbit was bled 3 days after each injection.

2.3. Western blotting

2.3.1. Rat brain

Three male Wistar rats (Clea Japan, Tokyo, Japan) weighing approximately 200 g were used. Under deep anesthesia with pentobarbital (80 mg/kg), the animals were decapitated. Approximately 50 mg of the striatum dissected from the brain was processed for a 10% SDS-PAGE as described previously (Tooyama and Kimura, 2000). Fractions electrophoresed were transferred to a nitrocellulose membrane, followed by blocking for 1 h with 0.1% bovine serum albumin dissolved in 10 mM phosphate buffer containing 0.3% Triton X-100. The membrane was incubated overnight with the best antiserum obtained, for 1 h with a peroxidase-labeled anti-rabbit IgG (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan; diluted 1:300), and for 5 min with West Pico Substrate (Pierce Biotechnology, IL, USA) to observe chemiluminescent signals.

2.3.2. Cell culture

Human embryonic kidney (HEK293) cells stably expressing cChAT or pChAT tagged with green fluorescent protein (GFP) or GFP alone were cultured, and soluble fractions of these cells were prepared as reported previously (Matsuo et al., 2005). Aliquots containing approximately 100 μg of protein were processed for a 7.5% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). After soaking for 1 h in a blocking reagent N-102 (Nippon Yushi, Osaka, Japan: diluted 1:5), the membranes were incubated for 16 h with one of the primary antibodies diluted with 50 mM Tris–HCl-buffered saline containing 0.05% Tween-20 (pH 7.4) at 4 °C. The primary antibodies used were anti-GFP antibody (mouse monoclonal, Roche, diluted 1:2500), pChAT antiserum (rabbit, diluted 1:5000)(Tooyama and Kimura, 2000), and the antiserum produced here against the cChAT peptide encoded by exons 7 and 8. West Pico Substrate was used as described above to detect chemiluminescent signals on the membranes.

2.4. Immunohistochemistry

Three male Wistar rats weighing 200–250 g were used. Under pentobarbital anesthesia (80 mg/kg), the animals were fixed by perfusion to process for immunohistochemistry as described previously (Tooyama and Kimura, 2000). Cryostat 20 μm-thick sections of the brain and trigeminal ganglion were incubated overnight in a free-floating state with the cChAT antiserum (diluted 1:20,000) or pChAT antiserum (diluted 1:20,000), for 3 h with biotinylated anti-rabbit IgG (diluted 1:5000, Vector Laboratories, CA), and for 1 h with the avidin–biotin-peroxidase complex (ABC Elite, 1:5000, Vector). Color was developed by reacting the sections for 20 min with 50 mM Tris–HCl buffer (pH 7.6) containing 0.02% 3,3′-diaminobenzidine, 0.005% H_2O_2 and 0.3%

Download English Version:

https://daneshyari.com/en/article/2202254

Download Persian Version:

https://daneshyari.com/article/2202254

Daneshyari.com