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DIFFERENTIAL EXPRESSION OF POLYCYTOSINE-BINDING PROTEIN ISOFORMS IN ADRENAL GLAND, LOCUS COERULEUS AND MIDBRAIN

N. M. BOSCHI,[a]* K. TAKEUCHI,[a,b] C. STERLING[a] AND A. W. TANK[a]

[a] *Department of Pharmacology & Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, United States*

[b] *Department of Psychiatry, North Rias Hospital, Monzen 1-151-1, Kuji, Iwate 0280021, Japan*

Abstract—Polycytosine-binding proteins (PCBPs) are RNA-binding proteins that participate in post-transcriptional control pathways. Among the diverse functions of these proteins is the interaction with a 27 nucleotide pyrimidine-rich domain within the 3′UTR of tyrosine hydroxylase (TH) mRNA. Mutations to this domain result in decreased stability of TH mRNA and loss of cAMP-mediated activation of TH mRNA translation. PCBPs are hypothesized to play key roles in these regulatory mechanisms. In order to further test this hypothesis, we examined the tissue distribution of PCBPs in catecholaminergic cells. Initial studies demonstrated that proteins from catecholaminergic tissues bind to TH mRNA 3′UTR sequences and these proteins have an apparent Mr of ~44 kDa, which is close to the molecular sizes for PCBPs. Fluorescent immunohistochemistry and confocal microscopy was used to analyze the distribution of PCBP isoforms in TH-positive cells of the rat midbrain, locus coeruleus, and adrenal gland. Our results suggest that: (1) PCBP2 is the predominant isoform in TH-positive cells of the rat midbrain; (2) PCBP3 is the predominant isoform in TH-positive cells of the locus coeruleus; and (3) PCBP1 is the predominant isoform in the adrenal medulla. The localization of PCBP proteins to TH-positive cells in these catecholaminergic tissues is consistent with the hypothesis that PCBPs play a role in the regulation of TH expression.

Key words: tyrosine hydroxylase, polycytosine-binding proteins, midbrain, locus coeruleus, adrenal medulla.

INTRODUCTION

Polycytosine-binding proteins (PCBPs) are a family of RNA-binding proteins characterized by high affinity and sequence-specific interactions with single-stranded RNA or DNA rich in cytosines (see (Makeyev and Liebhaber, 2002; Choi et al., 2009) for reviews). They are composed of two subsets in mammalian cells: hnRNP K/J and PCBPs, which are also referred to as alpha-complex proteins (alpha-CPs) or hnRNP Es. There are four genes encoding PCBPs and alternatively spliced forms of PCBP2 and PCBP4 are also expressed; hnRNP K/J is encoded by a separate gene. All PCBPs and hnRNP K/J have similar structures, comprising three KH (hnRNP K homology) domains that mediate RNA binding and a nonconserved variable region between the two N-terminal KH domains and the third C-terminal KH domain. Functionally, PCBPs are involved in almost every aspect of RNA metabolism, including transcriptional control, nuclear RNA splicing, mRNA translational control and mRNA stability. Very little is known about their role in the nervous system; however, they are thought to participate in regulating the transcription of the mu opioid receptor gene (Ko and Loh, 2005; Choi et al., 2007, 2009) and the stability and translation of tyrosine hydroxylase (TH) mRNA (Paulding and Czyzyk-Krzeska, 1999; Xu et al., 2009).

PCBPs were first implicated in TH mRNA regulation by Czyzyk-Krzeska et al. (1994), Czyzyk-Krzeska and Beresh (1996), Paulding and Czyzyk-Krzeska (1999), who showed that these proteins bind to a 27-bp pyrimidine-rich sequence within the TH mRNA 3′UTR. This binding is associated with regulation of TH mRNA stability in PC12 cells. More recently, work from our laboratory has implicated PCBP2 binding to these same pyrimidine-rich TH mRNA sequences in the regulation of TH mRNA translation in midbrain dopamine neurons (Chen et al., 2008; Xu et al., 2009). Taken together, these results suggest that PCBPs may play an important role in post-transcriptional regulation of TH mRNA. However, there is no information about whether these proteins are expressed in TH-positive cells in the adrenal gland or brain; hence, it remains unclear whether these proteins are viable candidates for regulating TH mRNA *in vivo*.

In this report, we have used biochemical and immunohistochemical assays to test which of the PCBP isoforms are expressed in the adrenal medulla, norepinephrine-expressing cells of the locus coeruleus, and dopamine-expressing cells of the midbrain in the rat. Our results suggest that although all PCBP isoforms

*Corresponding author. Address: Department of Pharmacology & Physiology, Box 711, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, United States. Tel: +1-585-275-1681; fax: +1-585-273-2652.
E-mail address: nboschi@me.com (N. M. Boschi).

Abbreviations: ENA, European Nucleotide Archive; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KH domain, hnRNP K homology domain; NGS, normal goat serum; PBS, phosphate-buffered saline; PBS-Tx, Triton-X-100 dissolved in PBS; PCBP, polycytosine-binding protein; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TH, tyrosine hydroxylase.

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are expressed to varying degrees in these tissues, PCBP1 is the predominant isoform expressed in the adrenal medulla, PCBP2 is the predominant form in midbrain TH-expressing neurons, and PCBP3 is the predominant form in the locus coeruleus.

EXPERIMENTAL PROCEDURES

Treatment of rats

Male Sprague–Dawley rats (175–250 g) were purchased from Charles-River (Willmington, MA, USA) and maintained on a 12-h light–dark cycle with free access to food and water. For biochemical analyses, adrenal glands were removed while the animals were anesthetized (using 150-mg sodium pentobarbital administered intraperitoneally) and adrenal medullae were dissected on ice. Brains were removed following anesthetization and decapitation, and locus coeruleus and midbrain regions were dissected as previously described (Sun et al., 2004; Radcliffe et al., 2009). All tissues were immediately frozen on dry ice and stored at −80 °C. For immunohistochemical analyses, rats were intracardially perfused with 200–400 ml 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS), pH 7.2. All procedures and drug administrations with rats were performed in accordance with the guidelines and approval of the University of Rochester Committee on Animal Resources.

RNA–protein binding assays and UV crosslinking analysis

These assays were performed as described by Xu et al. (2009). Briefly, the binding reactions (final volume of 30 μl) contained 10 μg S-100 cytoplasmic proteins, 3 μl of 10× binding buffer (100 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 500 mM KCl and 10 mM dithiothreitol) and 4-ng radiolabeled RNA probe expressing TH RNA 3′UTR sequences between the Kpn1 and Sph1 sites (see Fig. 1). The assays were carried out at 30 °C for 30 min. For competition experiments, proteins were incubated for 15 min with the indicated amount of competitor RNA prior to the addition of radiolabeled probe. After completion of the binding reaction, 1 μl of RNase T1 (50 units/μl) and 1 μl of heparin sulfate (1 μg/μl) were added sequentially to the reaction mixture for 10 min each at 30 °C. Electrophoresis of RNA–protein complexes was carried out using 6% nondenaturing polyacrylamide gels. After electrophoresis, the gels were dried and exposed to film. For UV-crosslinking of the RNA–protein complexes, binding reactions were performed as described above. The reaction mixtures were then placed on ice in a 96-well flat-bottom plate and exposed to UV light using a UV Crosslinker instrument (Stratagene Corporation, La Jolla, CA, USA) set at 1 J/cm^2 in Energy Mode. The crosslinked products were then digested for 20 min at 37 °C with RNase A (30 μg/ml) and RNase T1 (750 U/ml). The reaction products were separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and detected using autoradiography.

PCBP antisera

Antibodies for PCBP1, PCBP2 and PCBP3 were generated in rabbits by New England Peptide Corporation (Gardner, MA, USA) against the following peptide sequences: QHTISPLDLAK, amino acids 236–246 of rat PCBP1 (GenBank™ XP_002726454.2); RYSTGSDSASF, amino acids 195–205 of rat PCBP2 (GenBank™ NP_001013241.1); and GEKLPLH-SSEEAQN, amino acids 266–279 of rat PCBP3 (European Nucleotide Archive (ENA), UniProtKB/TrEMBL D4A1P3). Note that the sequence for rat PCBP3 in the ENA differs from that in GenBank, in that the amino acid sequence for the epitope to which the antibody was directed is deleted in the GenBank PCBP3 sequence. We confirmed that the PCBP3 sequence in the rats that we used in this study contained this epitope by amplifying PCBP3 from rat adrenal gland using reverse transcription polymerase chain reaction (RT-PCR) and sequencing the cloned PCR product. The sequence of this rat PCBP3 clone was identical to that reported in the ENA database. Antibody for rat PCBP4 was purchased from Abcam (Cambridge, MA, USA Product number: ab59534). Rabbit polyclonal antibody to TH was generated in our laboratory and has been used in numerous publications (Sun et al., 2003, 2004; Chen et al., 2008; Xu et al., 2009).

Extensive characterizations of these antisera were performed to determine appropriate dilutions for western analysis and immunohistochemistry. Using the optimal antiserum dilution, a single major protein band at the appropriate molecular size was detected on western blots using extracts derived from MN9D or PC12 cells. This major band was almost undetected using antiserum that was preabsorbed with either recombinant purified PCBPs or the peptide to which the antiserum was directed (see Fig. 2A for representative data). The blocking peptide for the PCBP4 antibody was purchased from Abcam.

Immunohistochemical assays

After perfusion, brain and adrenal glands were dissected and post-fixed in 4% paraformaldehyde, pH 7.2 for 1 h (adrenals) or 2 h (brains) at 4 °C. Tissue was then placed in 20% sucrose for 48 h at 4 °C and then frozen on dry ice. Sections (30 μm) were obtained using a freezing sliding microtome and stored in cryoprotectant at −20 °C until processed for immunohistochemical evaluation.

Double-immunofluorescence assays

Immunohistochemical analyses of tissue sections were carried out via a free-floating method. Briefly, tissue sections were rinsed 3–4 times to remove traces of cryoprotectant, permeabilized for 15 min with 0.4% Triton-X-100 dissolved in PBS (PBS-Tx), and blocked for 30 min in 10% normal goat serum (NGS) in PBS-Tx. Appropriate primary antibodies were diluted in 10% NGS/PBS-Tx (PCBP1 1:10,000, PCBP2 1:1000, PCBP3 1:10,000, PCBP4 1:1000, TH 1:40,000) and applied to