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Developmental regulation of Ubc9 in the rat nervous system

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The SUMO-conjugating enzyme Ubc9 is an essential enzyme in the SUMO (small ubiquitin-related modifier) protein modification system. Although sumoylation, covalent modification of cellular proteins by SUMO, is considered to regulate various cellular processes, and many substrates for sumoylation have been identified recently, the regulation of Ubc9 expression has not been examined in detail. We analyzed the expression of Ubc9 during rat brain development at the mRNA and protein levels. Northern and Western blot analyses revealed that expression of Ubc9 and SUMO-1 was developmentally regulated, while that of the ubiquitin-conjugating enzyme UbcH7 did not change so dramatically. *In situ* hybridization analysis revealed that the expression of *Ubc9* was high in neuronal stem cells and moderate in differentiated neurons at embryonic stages. In the adult brain, moderate expression was observed in subsets of neurons, such as the dentate granular neurons and pyramidal neurons in the hippocampal formation and the large pyramidal neurons in the cerebral cortex. These results suggest that the Ubc9-SUMO system might participate in the proliferation and differentiation of neuronal cells in the developing brain and in neuronal plasticity in the adult brain.

Keywords: Ubc9, SUMO, ubiquitin, brain development, *in situ* hybridization

INTRODUCTION

Modification of proteins by the covalent attachment of small polypeptides is an important regulatory mechanism. The best known molecule of this type is ubiquitin. Ubiquitination is critical for targeting proteins to be degraded by the proteasomes. At least three kinds of enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) are involved in the ubiquitination process (for review, see Hochstrasser, 1996). Presently, substrate specificity for ubiquitination appears to be decided by the interaction of the target protein with E2 and/or E3 enzymes. Ubiquitin is not the only molecular tag for protein modification. A family of small modifiers with sequence homology to ubiquitin, the SUMO (small ubiquitin-related modifier) family, has been identified, and it has been revealed that the conjugation pathway for SUMO ('sumoylation') is similar to ubiquitination and requires the E1 heterodimer AOS1/UBA2, a single E2 enzyme Ubc9, and a variety of E3 enzymes such as the PIAS family members (for recent reviews: see Dohmen, 2004; Johnson, 2004; Hay, 2005). Ubc9, with the assistance of an E3 enzyme, determines the substrate specificity. Recently, many substrates for sumoylation have been identified either individually or through proteomic approaches, and the number is still expanding (Dohmen, 2004; Li *et al*., 2004; Gocke *et al*., 2006; Vertegaal *et al*., 2006). Although sumoylation is considered to regulate various cellular processes such as nuclear transport, cell cycle progression, signal transduction and modulation of ubiquitination, its regulation has not been elucidated in detail. Ubc9 is the only E2-type enzyme critical for sumoylation and its expression

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Abbreviations: CP, cortical plate; DG, dentate gyrus; DIG, digoxigenin; EGL, external granular layer; IGL, internal granular layer; PCL, Purkinje cell layer; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone.

might regulate various processes, such as the balance between sumoylation and ubiquitination. Thus, we analyzed the spatio-temporal expression patterns of *Ubc9* mRNA and protein in developing and adult rat brain.

MATERIALS AND METHODS

Northern blot analysis. Poly(A)$^+$ RNA was isolated from embryonic day 13 (E13), E15, E18, E20, postnatal day 0 (P0), P4, P7, P14, P20 and 3-month-old rat brains (Wistar strain) using the Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA, USA). A DNA probe corresponding to the entire coding region of rat *Ubc9* gene was isolated by PCR and the ^{32}P-labeled probe was hybridized to a Northern blot filter containing 1 μg of poly(A)$^+$ RNA per lane. The filter was hybridized at 65ºC for 16 h in hybridization buffer and washed at room temp. in 1×SSC/0.1% SDS for 40 min and at 65ºC in 0.1×SSC/0.1% SDS for 1 h. The result was analyzed using a BioImage Analyzer (BAS 2000; Fuji Photo Film). The rat *elongation factor 1α (EF1α)* probe was used as an internal control.

Western blot analysis. Brain samples were prepared from whole brains of E13, E15, E18, E20, P7, P16, 6 month, and 1.5 year old rats. Brain tissues were homogenized in 10 mM Tris/HCl (pH 7.4), 1% SDS, 1 mM Na_3VO_4. The supernatants after centrifugation at 10000×g for 30 min were collected. Protein samples (40 μg/lane) were separated in 12.5% SDS polyacrylamide gels. The gels were then electrically blotted onto PVDF membranes and blocked with 5% non-fat milk. After incubation with a mouse monoclonal antibody against Ubc9 (1:250) (Cat. no. U61320, Transduction Laboratories, USA), a goat polyclonal antibody against Ubc9 (1:200) (Cat. no. sc-5231, Santa Cruz Biotechnology, USA), a mouse monoclonal antibody against SUMO-1 (1:100) (clone no. 21C7, Cat. no. 33-2400, Zymed, USA) or a monoclonal antibody against UbcH7 (1:250) (Cat. no. U66820, Transduction Laboratories), horseradish peroxidase-conjugated anti-mouse immunoglobulin was used as a secondary antibody. The signals were visualized by the chemiluminescence detection method.

***In situ* hybridization.** *In situ* detection of mRNAs using digoxigenin (DIG)-labeled probes was performed according to a method described previously (Takahashi *et al*., 2003). The purified DNA described above was used as a template for making RNA probes. Both sense and antisense RNAs were prepared by *in vitro* transcription using DIG-11-UDP (Roche, Germany) and T7 RNA polymerase and SP6 RNA polymerase, respectively. Brain tissues of E15, E18, E20, E22, P3, P7, P14, P21 and 3-month-old rats were fixed with 4% paraformaldehyde in phosphate buffer and processed as described below. Cryo-cut sections were treated with 0.2 M HCl and proteinase K (1 μg/ml), and then hybridized with 0.5 μg/ml of DIG-labeled RNA probes in hybridization buffer (5×SSC, 2% blocking reagent, 50% formamide) overnight at 65ºC. The sections were washed with 2×SSC containing 50% formamide for 1 h at 65ºC, followed by RNase A (20 μg/ml) treatment in 10 mM Tris/HCl (pH 8.0), 500 mM NaCl for 30 min at 37ºC. The sections were washed sequentially with 2×SSC and 0.2×SSC for 20 min at 65ºC. Thereafter, the sections were incubated with alkaline phosphatase-conjugated goat anti-DIG Fab' antibody (Roche, Germany) for 1 h at room temp. Colorimetric detection was performed by a standard immuno-alkaline phosphatase reaction, using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as the substrate.

RESULTS

Developmental expression of *Ubc9* mRNA and protein in rat brain

To examine the pattern of expression of *Ubc9* mRNA, Northern blot analysis was performed (Fig. 1A). Two transcripts of approx. 3.2 kb and 1.0 kb were detected in embryonic brains. The expression of the large transcript increased from E13 to E18 and thereafter decreased during development, while the small transcript was detected from the embryonic to the early postnatal stage, but not from the late postnatal to the adult stage (Fig. 1A, upper panel). The expression level of rat *elongation factor 1α* mRNA, analyzed as a control, did not change during development (Fig. 1A, lower panel).

To examine whether the expression level of *Ubc9* mRNA reflects the protein level, Western blot analysis was performed using two antibodies recognizing different epitopes of the Ubc9 protein (Fig. 1B). Both antibodies recognized an 18-kDa protein, which is the expected size of Ubc9. The amount of Ubc9 protein was developmentally regulated. The protein level was high during the embryonic stage, but decreased postnatally (Fig. 1B, right and left panels). Thus, the protein level was well correlated with the expression levels of the two transcripts of the *Ubc9* gene. We also investigated the expression of SUMO-1 protein (Fig. 1C, left panel). SUMO-1-conjugated proteins (Fig. 1C, indicated by an asterisk) were abundant at the embryonic stage, and the amount decreased during development. SUMO-1 monomer was also abundant at the embryonic stage (Fig. 1C, arrow). In contrast, the level of UbcH7

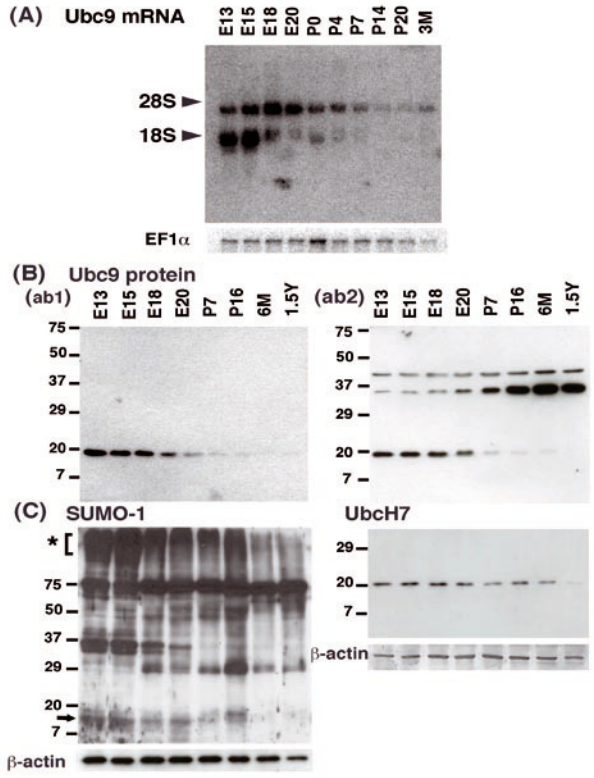

Figure 1. Expression of the *Ubc9* gene and Ubc9 protein in the developing and adult brain.
(A) Northern blot hybridization was performed using 1 μg of poly(A)$^+$ RNA from brains at various developmental stages. RNA isolated from embryonic day 13 (E13), E15, E18, E20, postnatal day 0 (P0), P4, P7, P14, P20 and 3-month-old (3M) rat brains was hybridized. Expression of Ubc9 transcripts was developmentally regulated (upper panel). The membrane was re-probed with a rat *elongation factor 1α* (*EF1α*) probe as an internal control. (B) Western blot analysis of Ubc9 protein during brain development. Homogenates of embryonic day 13 (E13), E15, E18, E20, postnatal day 7 (P7), P16, 6-month (6M) and 1.5-year (1.5Y) old rat brains were analyzed. (ab1) A polyclonal antibody to Ubc9 recognized an 18-kDa band and the level of which decreased during development (left panel). (ab2) Another antibody to Ubc9 also recognized this 18-kDa band (right panel). Both 35-kDa and 43-kDa bands were non-specifically detected by this antibody. (C) Western blot analysis of SUMO-1 and UbcH7 proteins during brain development. The membranes used in part B were reacted with other antibodies in part C (Ubc9 ab1 and SUMO-1; Ubc9 and UbcH7). A monoclonal antibody to SUMO-1 recognized many proteins, including SUMO-1 conjugates (100–200 kDa in size, indicated by an asterisk) and SUMO-1 monomer (14 kDa in size, indicated by an arrow). SUMO-1 conjugates and monomer were more abundant in the embryonic brains. The 75 kDa band is most likely sumoylated RanGAP1 on the basis of other studies. The β-actin protein level was used as an internal control (lower panel). The level of UbcH7 protein did not decrease dramatically (right panel), contrasting with the significant decrease in Ubc9 level during development. β-actin protein expression was used as an internal control for UbcH7 and Ubc9(ab2) (lower panel).

protein, an E2 enzyme for ubiquitination, did not decrease so dramatically during brain development except in the 1.5-year-old (Fig. 1C, right panel).

Spatio-temporal expression of *Ubc9* gene

To reveal the spatial distribution of *Ubc9* transcript, we performed *in situ* hybridization in rat embryonic and postnatal brains.

Expression of *Ubc9* mRNA was detected in various regions, including the cerebral cortex, hippocampus, amygdala, septum, basal ganglia, thalamus, hypothalamus, midbrain, pons, cerebellum, medulla oblongata and spinal cord, at the embryonic stage, but its expression became restricted to specific regions postnatally. In the adult brain, moderate expression was detected in a subset of neurons in the cerebral cortex and hippocampus, but not in the cerebellum. No significant hybridization signal was detected in glial cells. Throughout development, the *Ubc9* sense probe did not give any significant signals (Fig. 2E). For that reason, we focused on the developmental change in expression in the cerebellum, hippocampus and cerebral cortex as follows.

Expression of *Ubc9* in the cerebellum

Ubc9 expression was detected in the cerebellar primordia at E18 (not shown). At E22, a high level of expression was observed in the cells of the external granular layer (EGL) and moderate expression was observed in the identifiable Purkinje neuron precursors (Fig. 2A). As the maturation of the cerebellum proceeded, the high level of expression continued in the external granular layer (EGL), but the expression disappeared in the Purkinje neurons (PCL). Moderate expression was detected in the granular neurons of the internal granular layer (IGL), which migrated from the external granular layer (Fig. 2B). From P21 to adult, *Ubc9* expression was barely detected, even in granular neurons (Fig. 2C).

Expression of *Ubc9* in the hippocampus

Ubc9 expression was detected in the hippocampal anlage at E15 (Fig. 2D, arrow). In addition to the high level of expression in the ventricular and subventricular zones of the future hippocampus, moderate expression was seen in the early generating neurons of the developing hippocampus (Fig. 2F, arrowheads). Postnatally, *Ubc9* expression was observed in the pyramidal neurons in the CA1-CA3 and hilar regions and the dentate granular neurons (DG) (Fig. 2G). The high level of expression in the pyramidal neurons of CA1 to hilus and the granular neurons in the dentate gyrus (DG) continued until adulthood (Fig. 2K), and the level of

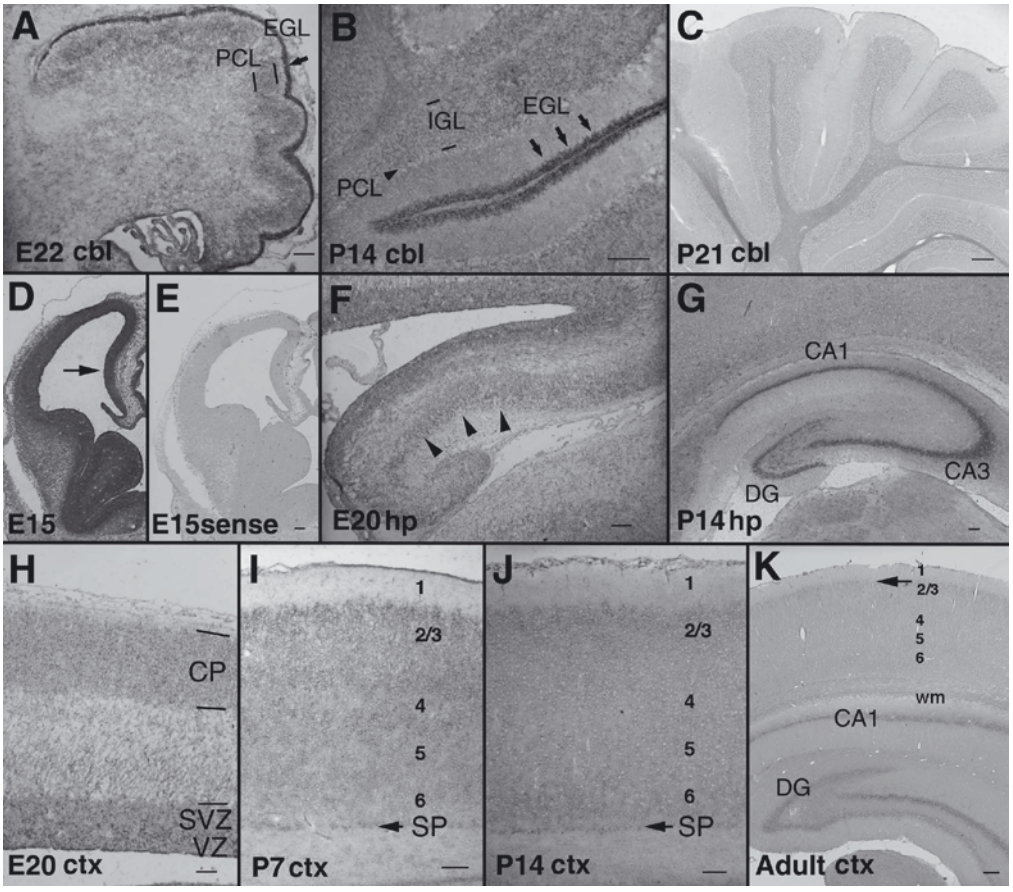

Figure 2. Expression of *Ubc9* gene in the developing cerebellum, hippocampus and cerebrum.
All except (E) were hybridized with antisense *Ubc9* RNA probe. No signal was detected in the section hybridized with a sense probe (E). Cerebellum (cbl) (A, B, C): intense signal for *Ubc9* was detected in the external granular layer (EGL) (arrow in A) and moderate signal was detected in the Purkinje cell layer (PCL) (A) at E22. Signal was observed in the external granular layer (EGL) and the internal granular layer (IGL) at P14 (B), but was barely detectable at P21 (C). Expression of *Ubc9* at E15 (D): intense signal was seen in the ventricular zone (VZ) of the developing cerebral cortex, basal ganglia and hippocampus. Moderate signal was also detected in the differentiated zone of this structure. Hippocampus (hp) (F, G): differentiated neurons of the hippocampus (arrowheads in F) as well as the cells of the ventricular zone expressed *Ubc9* (F). Neurons in the dentate gyrus (DG) and CA1-4 regions expressed *Ubc9* at P14 (G). Cerebral cortex (ctx) (H–K). Intense signal was detected in the ventricular zone (VZ) and subventricular zone (SVZ), and moderate signal was detected in the cortical plate (CP) of the developing cortex at E20. Signal was observed in the neurons of the cortical plate (CP) and subplate (SP) at P7 and P14. The expression level decreased in the adult cortex (K). cbl: cerebellum; hp: hippocampus; ctx: cerebral cortex; PCL: Purkinje cell layer; EGL: external granular layer; IGL: internal granular layer; VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate; SP: subplate, DG: dentate gyrus; WM: white matter. Scale bars in A, B, E, F and G, 100 μm; in C and K, 200 μm; in H, I and J, 50 μm.

expression was not different between these regions (Fig. 2K).

Expression of *Ubc9* in the cerebral cortex

Expression of *Ubc9* gene was detected in the ventricular and subventricular zones and the cortical plate of the cerebral cortex at E15 (Fig. 2D). The expression level in the ventricular and subventricular zones (VZ, SVZ) was higher than that in the cortical plate (CP). This pattern of expression continued until the late embryonic stage (Fig. 2H). Postnatally, the level of expression gradually decreased and moderate expression was localized to the cortical neurons of layers 2/3 and 5/6 and the subplate (SP) neurons (Fig. 2 I, J). In the adult, weak expression was detected in cortical large neurons of layer 2/3 (Fig. 2K, arrow). The expression level was not different between cortical areas (not shown).

DISCUSSION

Ubc9 is the sole specific E2-type conjugating enzyme for SUMO proteins (for reviews see: Dohmen, 2004; Johnson, 2004, Hay, 2005). Thus, sumoylation is dependent upon the expression of Ubc9. The expression of Ubc9 in mammals has been investigat-

ed in several studies. The tissue distributions of *Ubc9* mRNA and Ubc9 protein in adult rat, mouse and human were examined by Northern blot, RT-PCR and Western blot analyses (Hateboer *et al*., 1996; Wang *et al*., 1996; Watanabe *et al*., 1996; Yasugi & Howley, 1996; Loveys *et al*., 1997; Golebiowski *et al*., 2003). *Ubc9* mRNA was shown to be weakly expressed in adult rat, mouse and human brains. Among adult rat organs, Ubc9 protein expression in the brain was not so abundant compared with that in other tissues (Golebiowski *et al*., 2003). Although increasing attention is being paid to sumoylation in the nervous system (Martin *et al*., 2007; Scheschonka *et al*., 2007), the spatio-temporal expression of *Ubc9* has not been examined to date. Considering that Ubc9 has the ability to directly recognize substrate proteins, unlike other conjugating enzymes of ubiquitin-like proteins, and is the sole specific E2-like enzyme for sumoylation, the regulation of *Ubc9* expression should be an important issue. We have addressed this issue. Our analyses have revealed that *Ubc9* mRNA and Ubc9 protein are abundant in embryonic brains. In the embryonic stage SUMO-1 conjugates were also abundant and the changes in the levels of SUMO-1 conjugates were in accordance with those of Ubc9. Our analysis using *in situ* hybridization revealed high expression of *Ubc9* in the proliferating neuronal stem cell population residing in the ventricular zone, and moderate expression was detected in immature neurons in various regions of the developing brain. We also observed a high level of expression of the *Ubc9* gene in large cortical neurons and hippocampal neurons in the adult brain, which are considered to have high synaptic plasticity.

We discuss the relationship between the expression of Ubc9 and its interacting proteins below. Covalent modification of proteins by SUMO is considered to regulate various cellular processes such as nuclear transport, cell cycle progression, signal transduction and modulation of ubiquitination (for reviews, see Dohmen, 2004; Johnson, 2004; Hay, 2005). A large number of proteins have been shown to interact with Ubc9 in yeast two-hybrid assays. It was reported that topoisomerases I and II, enzymes essential for DNA replication and transcriptional regulation, interact with Ubc9 and are sumoylated (Mao *et al*., 2000a, 2000b). The topoisomerases are highly expressed in neuronal stem cells. The high expression of *Ubc9* in the ventricular zone of the developing brain revealed by our analysis is in accordance with the high expression levels of the topoisomerases in neuronal stem cells. Thus, it is reasonable to speculate that Ubc9 may participate in the proliferation of neuronal stem cells.

It has been reported that myoblast differentiation is regulated by Ubc9: myotube formation was impaired by *Ubc9* knockdown using siRNA (Riquelme *et al*., 2006a). The same scenario may be true for the involvement of Ubc9 in neuronal differentiation. The tumor suppressor protein p53, interacting with Ubc9, plays a regulatory role in the differentiation and apoptosis of neurons and oligodendrocytes in the central nervous system (Eizenberg *et al*., 1996; Gostissa *et al*., 1999; Rodriguez *et al*., 1999). The myocyte enhancer factor 2 (MEF2) family of transcription factors is implicated in the regulation of neuronal differentiation as well as muscle differentiation (Riquelme *et al*., 2006b; Shalizi *et al*., 2006). Recently, it has been reported that MEF2A is required for an early step in dendritic morphogenesis, and the transcriptional activity of MEF2 is repressed by sumoylation (Shalizi *et al*., 2006). Thus, the Ubc9/SUMO system might be important for differentiation and apoptosis *via* these molecules.

The modulator of transcription factor NFκB activity, IκB, also interacts with Ubc9 (Desterro *et al*., 1998). The Ubc9/SUMO system has been shown to be directly involved in the degradation of IκB, the loss of IκB leading to the activation and migration into the nucleus of NF-κB (Desterro *et al*., 1998; Yates & Gorecki, 2006). It is well known that NF-κB activation is regulated by the electrical activity within neurons and synaptic transmission between them (Mattson *et al*., 2001). The high expression of *Ubc9* in neurons, especially in the hippocampus and cerebral cortex of the adult brain, might reflect the activity of NF-κB related to neural plasticity in those neurons.

These results suggest that the Ubc9-SUMO system might participate in the proliferation and differentiation of neuronal cells in the developing brain and in neuronal plasticity in the adult brain.

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