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GABAA receptor subunit β1 is involved in the formation of protease-resistant prion protein in prion-infected neuroblastoma cells

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ABSTRACT

γ-Aminobutyric acid type A (GABAA) receptor β1 (gabrb1), a subunit of GABAA receptors involved in inhibitory effects on neurotransmission, was found to associate with the formation of protease-resistant prion protein in prion-infected neuroblastoma cells. Silencing of gabrb1 gene expression significantly decreased the abnormal prion protein level but paradoxically increased the normal prion protein level. Treatment with a gabrb1-specific inhibitor, salicylidene salicylhydrazide, dose-dependently decreased the abnormal prion protein level, but silencing of other GABAA receptor subunits' gene expression and treatments with the receptor antagonists and agonists did not. Therefore, gabrb1 involvement in abnormal prion protein formation is independent of GABAA receptors.

1. Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creuzfeldt–Jakob disease in humans, and bovine spongiform encephalopathy and scrapie in animals. These diseases are characterized by deposition of a partially protease-resistant abnormal isoform of prion protein (PrPres), which is the main component of the pathogen and which is converted from the normal cellular isoform of prion protein (PrPc) in the central nervous system and lymphoreticular system [1]. Cell biology of the biosynthesis and metabolism of PrPc and PrPres has been eagerly investigated in prion-infected cells [2,3] but has not been fully elucidated. Especially, endogenous factors involved in the formation of PrPres or the conformational change from PrPc into PrPres [4,5] remain enigmatic.

Using gene screening by the gene silencing technique with small interfering RNA (siRNA) or short hairpin RNA (shRNA) [6,7], we have sought endogenous factors affecting the metabolism of PrPres in prion-infected neuroblastoma cells. We report here a possible linkage of γ-aminobutyric acid A receptor β1 (gabrb1) with the formation of PrPres. In fact, gabrb1 is a subunit of γ-aminobutyric acid type A (GABAA) receptors responsible for most of the fast inhibitory synaptic transmission in mammalian brain [8]. Belonging to the ligand-gated ion channels, they are formed by the pentameric assembly of homologous subunits. Numerous GABAA receptor subunits have been identified (α1–6, β1–3, γ1–3, δ, π, ε, and θ), all of which are products of separate genes, and most GABAA receptors contain two α subunits, two β subunits and either one γ subunit or one δ subunit [8]. It has been well documented that GABAergic neurons are affected by prion infection [9–15], but it is not clear whether GABAA receptors are involved in PrPres formation. Therefore, to address this query, we performed gene silencing experiments for gabrb1 and other representative GABAA receptor subunits (α5, β3, γ2, and δ) as well as GABAA receptor modulating experiments using the antagonists and agonists in prion-infected cells.

2. Materials and methods

2.1. siRNAs and compounds

Double-stranded siRNAs for GABAA receptor subunits used for this study (Table 1) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Some GABAA receptor antagonists (bicuculline

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methiodide and picrotoxin) and agonists (GABA, muscimol, and isoguvacine hydrochloride) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Another agonist, pentobarbital, was purchased as a 5% (approximately 200 mM) solution of its sodium salt, nembutal, from Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). A specific inhibitor of GABAA receptor β1 subunit, salicylidene salicylhydrazide, was obtained from Tocris Bioscience (Missouri, USA).

2.2. shRNA expression vectors

The DNA fragments flanking 5′ *Bam*HI recognition sequence and 3′ *Hind*III recognition sequence, which corresponded to shRNA sequences for GABAA receptor subunits, were produced by annealing pairs of sense and antisense 64mer oligonucleotides, of which sense sequences were designed as follows: 5′-GATCC***GAgGGTGGAGTTCAtAAtAGG***CTTCCTGTCA***CCTGTTGTGAACTCCACCTTC***-TTTTTTA-3' targeting to nucleotides 663–683 of β1 subunit coding sequence; 5′-GATCC***ACCTGGTgTGAtAGAtGTTTG***CTTCCTGTCA***CAAACGTCTGTCATACCAGGT***TTTTTTA-3′ targeting to nucleotides 355–375 of γ2 subunit coding sequence; 5′-GATCC***GAATGGGCTgCTTtACtATCC***CTTCCTGTCA***GGATGGTGAAGTAGCCCATTC***TTTTTTA-3′ targeting to nucleotides 809–829 of γ2 subunit coding sequence; 5′-GATCC***ACATGGAgTACACtATGAtTG***CTTCCTGTCA***CAGTCATGGTGATTTCCATGT***TTTTTTA-3′ targeting to nucleotides 245–265 of δ subunit coding sequence. Bold italic letters and small bold italic letters, respectively denote target sites and mismatch-induced sites. The DNA fragments were ligated into a pBAsi-hH1 (Takara Bio Inc., Shiga, Japan) cut with *Bam*HI and *Hind*III. The ligated vectors were introduced into *Escherichia coli*; then plasmids of interest were harvested and sequenced.

2.3. Mutated gabrb1 expression vector

Mouse gabrb1 gene was cloned from a ready mouse brain cDNA library (Marathon; Takara Bio Inc.) using PCR with KOD-plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan). A PCR product was inserted into a pcDNA3.1 Myc/His expression vector (Invitrogen Corp.). The ligated vectors were introduced into *E. coli*; then plasmids of interest were harvested and sequenced. Mutations were induced by site-directed mutagenesis using PCR technique with primers of 5′-GATGCATCTGCAGC*g*c*G*t*G*TgGC*t*CT*t*GG*t*AT*a*AC*a*AC-GGTGCTG-3′ (small italic letters indicate silent mutations induced) and 5′-TGCAGATGCATCATAGTTGATCCA-3′. The PCR products were treated with *Dpn*I for digesting template plasmids and introduced directly into *E. coli*; then plasmids of interest were harvested and sequenced.

2.4. Gene silencing experiments

Mouse neuroblastoma N2a cells infected persistently with RML prion strain (ScN2a) or 22L prion strain (N167) were diluted to 10% or 15% confluence with Opti MEM I (Invitrogen Corp.) including 10% fetal bovine serum (FBS), and 2.4 ml each was seeded onto six-well plates. Transfection was performed on the next day of seeding. TransFectin (3.0 μl/well; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for the transfection of shRNA expression vectors, and siLentFect (3.0 μl/well, Bio-Rad Laboratories, Inc.) was used for transfection of double-stranded siRNAs. The amounts of vectors or siRNAs used in transfection were, respectively, 0.2 μg per well or 20 nM per well. Medium was changed on the day after transfection. Cells were harvested after washing with PBS 3 days after transfection.

Table 1
siRNAs and shRNAs used for this study.

GABAA receptor subunit examined	Nucleotide position of target region*	
	siRNA (catalog no.**)	shRNA
α5	311–335 (MSS201426)	
β1	820–844 (MSS204523)	663–683
β3	873–897 (MSS204527)	
β3 transcript variant 1	62–86 (MSS204528)	
γ2		355–375, 809–829
δ		245–265

* Nucleotide position in the coding sequence of each GABAA receptor subunit gene.
** siRNAs were obtained from Invitrogen Corp. (Carlsbad, CA, USA).

2.5. Rescue experiment

The ScN2a cells were diluted to 15% confluence with Opti MEM I including 10% FBS, and 2 ml each was seeded onto six-well plates. Transfection was performed on the day after seeding. TransFectin (2.0 μl/well) was used for the transfection of both mutated gabrb1 expression vector and double-stranded siRNA. The amounts of the vector and the siRNA used for transfection were, respectively, 0.8 μg per well and 5 nM per well. Medium was changed on the day after transfection. Cells were harvested after washing with PBS 3 days after transfection.

2.6. Immunoblotting

Cells were lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS) after rinsed with PBS, and debris was eliminated by centrifugation at 3000×*g* for 10 min at 4 °C. Protein contents of each sample were measured using a modified Lowry method [16] with Dc protein assay reagent (Bio-Rad Laboratories, Inc.) with bovine serum albumin as a standard. For PrPres detection, cell lysate containing the same protein amount was treated with 10 μg/ml of proteinase K for 30 min at 37 °C, and PrPres was pelleted by centrifugation at 20 000×*g* for 20 min at 4 °C. After denaturation in sample buffer by heating at 95 °C for 10 min, PrP was separated using SDS–PAGE and then transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). Subsequently, PrP was detected using a monoclonal antibody SAF83 as a primary antibody, which recognizes residues 126–164 of mouse PrP (1:5000; SPI-Bio, Massy, France), and an alkaline phosphatase-conjugated goat anti-mouse antibody (1:20 000; Promega Corp., Madison, WI, USA) as a secondary antibody. Immunoreactivity was visualized using CDP-Star detection reagent (Amersham, Piscataway, NJ, USA) and was analyzed densitometrically using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). To check the sample integrity, protein levels of GAPDH and β-actin were analyzed in the same samples used for PrPres detection.

2.7. Quantification of mRNA level

Cells were lysed with RNAiso-plus reagent (Takara Bio Inc.). Total RNA was extracted using FastPure RNA (Takara Bio Inc.). Poly A^+ RNA was purified from total RNA using an isolation kit (MicroFast Track MAG micro mRNA; Invitrogen Corp.). In addition, cDNA was synthesized with first strand cDNA synthesis kit (Takara Bio Inc.). The mRNA level was measured by real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.) or using TaqMan probe with gene expression assay master mix for gabrb1 (Mm00433461_m1; Applied Biosystems). Fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as an internal control.

2.8. Statistical analyses

Statistical significance was analyzed using one-way analysis of variance followed by the Tukey–Kramer post-hoc test for multiple