Abnormal N-Glycosylation of a Novel Missense Creatine Transporter Mutant, G561R, Associated with Cerebral Creatine Deficiency Syndromes Alters Transporter Activity and Localization

Tatsuki Uemura, Shingo Ito, Yusuke Ohta, Masanori Tachikawa, Takahito Wada, Tetsuya Terasaki, and Sumio Ohtsuki

Department of Pharmaceutical Microbiology, Graduate School of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-hommachi, Chuo-ku, Kumamoto 862–0973, Japan; AMED-CREST, Japan Agency for Medical Research and Development; 1–7 Otemachi, Chiyoda-ku, Tokyo 100–0004, Japan; Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University; 6–3 Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan; and Department of Medical Ethics and Medical Genetics, Graduate School of Medicine, Kyoto University; Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606–8501, Japan.

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Cerebral creatine deficiency syndromes (CCDSs) are caused by loss-of-function mutations in creatine transporter (CRT, SLC6A8), which transports creatine at the blood–brain barrier and into neurons of the central nervous system (CNS). This results in low cerebral creatine levels, and patients exhibit mental retardation, poor language skills and epilepsy. We identified a novel human CRT gene missense mutation (c.1681 G>C, G561R) in Japanese CCDSs patients. The purpose of the present study was to evaluate the reduction of creatine transport in G561R-mutant CRT-expressing 293 cells, and to clarify the mechanism of its functional attenuation. G561R-mutant CRT exhibited greatly reduced creatine transport activity compared to wild-type CRT (WT-CRT) when expressed in 293 cells. Also, the mutant protein is localized mainly in intracellular membrane fraction, while WT-CRT is localized in plasma membrane. Western blot analysis revealed a 68kDa band of WT-CRT protein in plasma membrane fraction, while G561R-mutant CRT protein predominantly showed bands at 55, 110 and 165kDa in crude membrane fraction. The bands of both WT-CRT and G561R-mutant CRT were shifted to 50kDa by N-glycosidase treatment. Our results suggest that the functional impairment of G561R-mutant CRT was probably caused by incomplete N-linked glycosylation due to misfolding during protein maturation, leading to oligomer formation and changes of cellular localization.

Key words  cerebral creatine deficiency syndromes; creatine; creatine transporter; N-linked glycosylation

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structure, proper folding, trafficking and transport function of SLC transporters.\textsuperscript{24–26} CRT has two N-glycosylation sites (Asn192 and Asn197), and deletion of either or both of them impairs CRT trafficking to the plasma membrane.\textsuperscript{39} Therefore, it is plausible that G561R-CRT missense mutation causes failure of CRT maturation due to protein misfolding and abnormal N-glycosylation, leading to loss of proper trafficking. However, it is important to establish the precise mechanism in order to find potential therapeutic targets for CCDSs. To investigate the transport and localization of the G561R-CRT mutant in this study, we chose 293 cells, which have been used in previous CRT mutation studies,\textsuperscript{30} probably because CRT is predominantly expressed in the kidney and it is easy to establish stable transfected 293 cell lines.

The purpose of the present study, therefore, was to characterize the functional attenuation of CRT with G561R mutation, and to examine the effect of G561R mutation on the subcellular localization and glycosylation of CRT by using 293 cells stably expressing the mutant.

MATERIALS AND METHODS

Reagents and Antibodies \textsuperscript{[14]}C]Creatine (55 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Anti-CRT antibody was produced as previously described.\textsuperscript{61} Anti-Flag antibody was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Peptide:N-glycosidase F (PNGase F) was purchased from New England Biolabs (Roche, Basel, Switzerland). All other chemicals were commercial products of analytical grade.

Construction of 293 Cells Stably Expressing N-3x Flag Tagged Human CRT The G1681C mutant of human CRT was generated with a site-directed mutagenesis kit according to the manufacturer’s protocol.\textsuperscript{16} Anti-Flag antibody was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Peptide:N-glycosidase F (PNGase F) was purchased from New England Biolabs (Roche, Basel, Switzerland). All other chemicals were commercial products of analytical grade.

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Functional Characterization of G561R-Mutant CRT Creatine transport by G561R-mutant CRT was examined in 293 cells stably expressing wild-type or G561R-mutant CRT were seeded on poly-d-lysine-coated 24-well plates (Iwaki, Shiga, Japan). The open reading frames (ORFs) of human wild-type CRT and G1681C mutant CRT were subcloned into pEBMutil puro vector (Wako), which includes an N-terminal 3x Flag tag gene. Transfections of plasmid DNA were performed with ScreenFectA (Wako) according to the manufacturer’s recommendations. The cells were cultured in the presence of 2.0 µg/mL puromycin for 1 week. The puromycin-selected cells were cultured on culture dishes at 37°C in an atmosphere of 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 70 mg/L benzylpenicillin, 100 mg/L streptomycin, 2 µg/mL puromycin, and 10% fetal bovine serum.

\[ ^{[14]}\text{C} \text{Creatine Uptake by 293 Cells} \]

293 cells stably expressing wild-type or G561R-mutant CRT were seeded on poly-l-lysine-coated 24-well plates (Iwaki, Shiga, Japan) at a density of 1.0×10\textsuperscript{4} cells/well and cultured for 48 h at 37°C. The uptake procedure and the estimation of cellular uptake were performed as described elsewhere.\textsuperscript{29}

Immunohistochemistry 293 cells stably expressing wild-type or G561R-mutant CRT were cultured on poly-l-lysine-coated slide glasses for 2d. The cells were fixed with 4% paraformaldehyde for 10 min and then treated with solution containing methanol (90% (v/v)), acetic acid (5% (v/v)), and MilliQ water (5% (v/v)). After blocking, cells were incubated with anti-CRT antibody at 4°C for 16 h, then washed with phosphate buffered saline (PBS), incubated with Alexa Fluor 546-conjugated secondary antibody for 30 min, and further incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Photographs were taken using a confocal laser scanning microscope (LSM, Zeiss, Oberkochen, DE, U.S.A.).

Western Blot Analysis Cytosol, crude membrane fraction and plasma membrane fraction of 293 cells stably expressing human CRT were separated with a Plasma Membrane Protein Extraction Kit (Bio Vision, Mountain View, CA, U.S.A.) according to the manufacturer’s protocol. Amounts of proteins in each fraction were determined with a bicinchoninic acid (BCA) protein assay kit (Bio-rad, Richmond, CA, U.S.A.) using bovine serum albumin (BSA) as a standard. Samples were incubated with sodium dodecyl sulfate (SDS) sample buffer containing 2-mercaptoethanol (2-ME) for 30 min at 37°C. Protein samples (5 µg) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and bands were electrotransferred to polyvinylidene difluoride membranes (Bio-rad). After incubation with blocking buffer (5% skimmed milk in 25 mm Tris–HCl (pH 8.0), 125 mm NaCl, 0.1% Tween 20) for 1 h at room temperature, membranes were incubated with anti-Flag (Wako) or anti-β-actin antibodies at 4°C for 16 h. The membranes were washed three times with TTBS buffer (25 mm Tris–HCl (pH 8.0), 125 mm NaCl, 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated antibody. Signals were visualized with an enhanced chemiluminescence kit (TaKaRa) and detected with an imager (Omega LumIG Imaging System, Aplegen Inc., San Francisco, CA, U.S.A.).

Deglycosidation For glycosidase digestion, 5 µg protein of cytosol, crude membrane or plasma membrane fraction was incubated with N-glycosidase F (1 U, Roche) in SDS sample buffer (0.25 mol/L Tris–HCl, 0.02% (w/v) bromophenol blue (BBP), 8% (w/v) SDS, 40% (w/v) glycerol, 20 vol% 2-ME, pH 6.8; Wako) for 17 h at 37°C. After digestion, samples were subjected to Western blot analysis as described above.

Statistical Analysis Unless otherwise indicated, all data are presented as the mean±standard error of the mean (S.E.M.). An unpaired, two-tailed Student’s t-test was used to determine the significance of differences between means of two groups. One-way ANOVA followed by Dunnett’s test was used to assess the statistical significance of differences among means of more than two groups.

RESULTS

Functional Characterization of G561R-Mutant CRT Creatine transport by G561R-mutant CRT was examined in 293 cells stably expressing N-3xFlag tagged G561R-mutant CRT, compared with cells stably expressing wild-type (WT) CRT. \textsuperscript{[14]}C]Creatine uptake by the mutant was significantly less than that by WT-CRT at all examined time points up to 40 min except for 0.25 min (Fig. 1). Furthermore, uptake by cells expressing the mutant was not significantly different from that by mock cells up to 10 min. At 40 min, the uptake of \textsuperscript{[14]}C] creatine by cells expressing G561R-mutant CRT was 1.32-fold greater than that by mock cells, while cells expressing WT-CRT exhibited 1.97-fold greater uptake than mock cells.

Initial creatine uptake clearance was calculated from the slope of \textsuperscript{[14]}C]creatine uptake up to 20 min (Fig. 1A), because the uptake of creatine by WT-CRT and G561R-mutant CRT expressing cells increased linearly up to 20 min (r=0.985 and 0.950, respectively, determined by linear regression analysis). The values of initial \textsuperscript{[14]}C]creatine uptake clearance by cells
expressing WT- and G561R-mutant CRT were determined to be 8.18 and 4.71 µL/mg protein/min, respectively (Fig. 1B). Creatine uptake clearance by exogenous CRT was estimated by subtracting that by mock cells (3.46 µL/mg protein/min) from that by either WT- or G561R-mutant CRT. The [14C]-creatinine uptake rates by exogenous WT- and G561R-mutant CRT were estimated to be 4.72 and 1.25 µL/mg protein/min, respectively. This suggests that creatine transport activity in 293 cells was strongly attenuated by the G561R mutation.

Creatine transport by CRT is saturable and Na\(^+\)-Cl\(^-\)-dependent. Therefore, to clarify the transport characteristics of G561R-mutant CRT, we further examined creatine uptake by WT- and mutant-CRT expressing cells in the presence and absence of unlabeled creatine or Na\(^+\) or Cl\(^-\) at 30 min, when the creatine uptake by WT- and mutant-CRT expressing cells was significantly greater than that by the mock cells (open bars in Fig. 1C). The uptake of [14C]creatinine by cells expressing WT- and G561R-mutant CRT, as well as mock cells, at 30 min was almost completely suppressed in the presence of excess unlabeled creatine, by 97.1 and 95.8%, respectively (Fig. 1C). The uptake of [14C]creatinine by WT- and G561R-mutant CRT at 30 min was reduced in the Na\(^+\) free condition by 97.1 and 96.4%, respectively, and in the Cl\(^-\) free condition by 95.4 and 95.2%, respectively (Fig. 1C). Uptake by mock cells was also suppressed by 94.2 and 94.6% in Na\(^+\) free and Cl\(^-\) free conditions, respectively. These results suggest that creatine transport by the exogenous CRT was saturable and Na\(^+\)-Cl\(^-\)-dependent, like that of endogenous CRT.

**Subcellular Localization of WT- and G561R-Mutant CRT Protein in 293 Cells** CRT functions as a transporter for creatine at the plasma membrane. To investigate whether G561R-missense mutation alters the localization of CRT in 293 cells, we examined the localization of CRT protein in WT- and G561R-CRT by means of immunohistochemistry using anti-CRT antibody (Fig. 2). The WT-CRT protein was predominantly expressed at the plasma membrane of 293 cells. In contrast, G561R-mutant CRT was visualized as dots in the intracellular compartment. This result suggests that G561R-missense mutation alters the subcellular localization of CRT protein in 293 cells.

**Expression of WT- and G561R-Mutant CRT Protein in Subcellular Fractions of 293 Cells** To examine the subcellular expression of exogenous G561R-mutant CRT protein, cells were fractionated into cytosol, crude membrane and plasma membrane fractions, and protein expression of exogenous CRT in each fraction was determined by Western blot analysis using anti-Flag antibody (Fig. 3). As shown in Fig. 3A, WT-CRT was predominantly detected at the plasma membrane. To investigate whether the exogenous G561R-mutant CRT proteins were predominantly localized in the plasma membrane fractions, and protein expression of exogenous CRT in each fraction was determined by Western blot analysis using anti-Flag antibody. As shown in Fig. 3A, WT-CRT was predominantly detected at 68 kDa and faintly detected at 179 kDa in plasma membrane fraction, which is consistent with the immunohistochemical results shown in Fig. 2. Total expression of G561R-mutant CRT was greater than that of WT-CRT. However, G561R-mutant CRT was detected at 55, 110 and 165 kDa in both the crude membrane and plasma membrane fractions (Fig. 3A). As shown in Fig. 3B, which was exposed for a shorter time to avoid signal saturation, the band intensities were greater in the crude membrane fraction than in the plasma membrane fraction, suggesting G561R-mutant CRT proteins were predominantly localized in intracellular membranes. The larger-molecular-size bands appear to be oligomers of 55 kDa protein. These results suggest that G561R-missense mutation increases intracellular localization of CRT.
levels of CRT protein, but induces protein structure changes and oligomerization. The band intensity at around 65 kDa of G561R-mutant CRT in plasma membrane fraction was greater than that in crude membrane fraction, suggesting that G561R-mutant CRT of similar molecular size to WT-CRT was expressed at the plasma membrane of 293 cells expressing the mutant CRT (Fig. 3).

**Abnormal N-Glycosylation of G561R-Mutant CRT in 293 Cells** To examine whether G561R-mutant of CRT undergoes abnormal N-glycosylation in 293 cells, the proteins in cytosol, crude membrane fraction and plasma membrane fraction were treated with N-glycosidase F to remove N-linked oligosaccharides from glycoproteins. After this treatment, both the band at 68 kDa of WT-CRT and that of G561R-mutant CRT at 55 kDa in the crude and plasma membrane fractions were shifted to 50 kDa (Fig. 4). This result suggests that the 68 and 55 kDa CRT proteins were differently N-glycosylated.

In Fig. 3, several bands of G561R-mutant CRT protein were observed in the crude membrane and plasma membrane fractions. In contrast, a single band at 55 kDa of G561R-mutant CRT was detected in the crude membrane and plasma membrane fractions treated with N-glycosidase F or control for 17 h.
at 37°C (Fig. 4). This result suggests that protein complexes or oligomers of G561R-CRT dissociated during the treatment.

DISCUSSION

The present study demonstrated that a novel CRT missense mutation in exon 12 of the SLC6A8 gene (c.1681G>C; p.G561R) causes suppression of creatine transport activity. G561R-mutant CRT is expressed predominantly in the intracellular compartment, whereas WT-CRT is localized at the plasma membrane. The G561R missense mutation in CRT alters N-glycosylation and promotes oligomer formation in intracellular organelle membranes. Therefore, our present findings suggest that G561R-missense mutation in CRT suppresses creatine transport due to defects in protein folding, N-glycosylation, and trafficking to the plasma membrane.

Patients with G561R-missense mutation in CRT showed a marked reduction of brain creatine level measured by \(^1\text{H}-\text{MRS}^{22}\). The physiological role of CRT expressed at the BBB is to supply creatine to the brain. Our present findings show that G561R-mutant CRT-expressing cells exhibit markedly reduced creatine uptake compared to WT-CRT-expressing cells, even though the total expression of exogenous CRT was greater in the former cells (Figs. 1, 3). Our in vitro study suggested that initial creatine uptake clearance by G561R-mutant CRT was reduced by 73% compared to that in WT-CRT (Fig. 1B). We previously reported that the brain creatine level in CCDSs patients was below the detection limit of \(^1\text{H}-\text{MRS}^{22}\). Previous study has shown that the brain creatine level was about 5 mmol/L in the gray matter of healthy persons and detection limit of brain creatine was less than 0.8 mmol/L.\(^{32}\) Thus, the brain creatine level in patients with G561R-CRT mutation appears to be reduced by at least 84% compared to that in healthy persons. Therefore, the reduction of transport activity by G561R-mutant CRT appears to be comparable with the reduction of creatine concentration in the brain of CCDSs patients with G561R-CRT mutation. Thus, we conclude that G561R missense mutation in CRT attenuates creatine transport activity at the BBB, resulting in a reduction of brain creatine level due to suppression of creatine supply to the brain across the BBB.

We found that G561R-mutant CRT is localized mainly in intracellular membranes (Figs. 2, 3). The intracellular localization of the transporter was examined using N-3x Flag tagged-protein. Previous studies have shown that N-terminally tagged CRT is predominantly localized at the plasma membrane,\(^{39}\) as is untagged CRT.\(^{35}\) Therefore, it seems plausible that the Flag-tag at the N-terminus would not affect the localization of WT-CRT, although the possibility that the N-terminal Flag-tag altered the localization of CRT cannot be completely excluded. Also, further study would be desirable to establish the localization of untagged G561R-mutant CRT.

We also found that the molecular weight of G561R-mutant CRT is about 13 kDa smaller than that of WT-CRT (Fig. 3). N-Glycosylation plays an important role in the regulation of transporter and channel functions, and in trafficking to the plasma membrane.\(^{25,29,34-36}\) Several reports demonstrate that CRT is N-glycosylated in at least two sites.\(^{30,37}\) N-Glycosidase F treatment caused a band shift of WT-CRT from 68 to 50 kDa, indicating that WT-CRT was N-glycosylated (Fig. 4). This is consistent with previous reports that treatment with N-glycosidase F or tunicamycin reduced the molecular weight of bovine and human creatine transporter expressed in 293 cells.\(^{30,37}\) The molecular size of G561R-mutant CRT was 55 kDa, which smaller than WT-CRT (Fig. 3). After N-glycosidase treatment, the molecular size of G561R-mutant CRT became the same as that of WT-CRT at 50 kDa. This suggests that the N-glycosylated G561R-mutant CRT was an immature form. However, the possibility that post-translational modification of CRT is different at the BBB and in neurons cannot be excluded, and further study will be needed to examine this issue.

N-Glycosylation of protein is a post-translational process, which is initiated within the endoplasmic reticulum. Properly folded and N-glycosylated protein is then exported to Golgi apparatus, where \(\text{Man}_{4}(\text{GlcNAc})_{2}\) oligosaccharide chains are added, and after further processing correctly decorated proteins are trafficked to the cell surface.\(^{24}\) Our present findings suggest that G561R-mutant CRT formed dimers and trimers in the intracellular membrane, in contrast to WT-CRT, which is present as a monomer in the plasma membrane (Fig. 4). Thus, it seems likely that G561R-missense mutation alters the protein folding of CRT and causes aberrant glycosylation. The expression level of G561R-CRT was higher than that in WT-CRT (Fig. 3). This may be due to accumulation of the misfolded G561R-CRT protein in intracellular membrane.

CRT is classified into the Na\(^+\)-Cl\(^-\)-dependent neurotransmitter transporter family. It was reported that Na\(^+\)-Cl\(^-\)-dependent neurotransmitter transporters such as human serotonin transporter\(^{50}\) and human dopamine transporter form oligomers,\(^{39}\) and proper oligomer formation of Na\(^+\)-Cl\(^-\)-dependent neurotransmitter transporters is required to pass the endoplasmic reticulum quality control mechanisms for trafficking to the plasma membrane.\(^{24}\) In the present study, oligomers might have remained partially intact under the mild denaturing conditions used to prepare plasma membrane fraction (37°C for 30 min), and this would be consistent with the faint band at around 179 kDa in Western blot analysis of the plasma membrane fraction of WT-CRT.

It was reported that the 11 and 12th transmembrane domains participate in oligomerization of Na\(^+\)-Cl\(^-\)-dependent neurotransmitter transporters such as human serotonin transporter.\(^{50}\) The G561R CRT missense appears to be localized at the 12th transmembrane domain. The alteration of G to R at CRT561 introduces a positive charge at the 12th transmembrane domain. Therefore, alteration of the electrostatic interaction of the 12th transmembrane domain may cause the improper protein folding of G561R-mutant CRT. Several CRT missense mutations associated with CCDSs have been reported to exhibit reduced creatine transport activity and smaller molecular size than WT-CRT.\(^{30}\) Thus, it is possible that the reduction of creatine transport by other CRT missense mutations may also be caused by defects of protein folding and/or trafficking.

Western blot analysis showed that G561R-CRT protein is expressed mainly in crude membrane fraction, as well as in the plasma membrane, but immunohistochemical analysis did not confirm protein expression of G561R-CRT in plasma membrane (Figs. 2, 3). Fractionation with the Biovision kit or sucrose density gradient method is a useful technique to enrich plasma membrane proteins, but the resulting fraction can be contaminated with proteins from other organelle membranes.
creatinine transport at the BBB and into neurons, resulting in depletion of cerebral creatine due to inadequate CRT-mediated energy homeostasis, G561R-mutant CRT likely causes chronic transport activity as a result of defective protein trafficking missense mutant of CRT, G561R, shows impaired creatine transport activity. Furthermore, the creatine uptake behavior of mutant CRT-expressing cells indicates that creatine uptake by exogenous G561R-mutant CRT was suppressed by excess unlabeled creatine and under Na$^+$-free conditions in the same manner as in the case of endogenous CRT. Therefore, the characteristics of creatine transport by G561R-mutant CRT appear to be similar to those of endogenous CRT (Fig. 1). Exogenous CRT of similar molecular size (about 65 kDa) to that of WT-CRT was detected in plasma membrane of G561R-mutant CRT-expressing cells, and may exhibit creatine transport activity to some extent. Impaired membrane trafficking of bile salt export pump (BSEP/ABCB11) was reported to be caused by missense mutations, but these BSEP mutants retain transport function. It was also reported that 4-phenylbutyrate, an U.S. Food and Drug Administration (FDA)-approved drug for urea cycle disorders, enhances the cell surface expression and transport capacity of mutated BSEP, thereby improving liver function and relieving pruritus in patients with progressive familial intrahepatic cholestasis type 2. Thus, a compound that modulates a molecular chaperone involved in CRT protein folding and/or transport of creatine analogues via a CRT-independent pathway could be a therapeutic target for CCDSs with G561R-mutant CRT missense mutation or other CRT missense mutations that exhibit altered protein folding and/or trafficking.

In conclusion, our present findings suggest that a novel missense mutant of CRT, G561R, shows impaired creatine transport activity as a result of defective protein trafficking to plasma membrane due to protein misfolding and altered N-glycosylation. Since creatine plays a pivotal role in brain energy homeostasis, G561R-mutant CRT likely causes chronic depletion of cerebral creatine due to inadequate CRT-mediated creatine transport at the BBB and into neurons, resulting in neurodevelopmental disorders.

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Conflict of Interest The authors declare no conflict of interest.

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