ORIGINAL ARTICLE

EGFP Tags Affect Cellular Localization of ATP7B Mutants

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SUMMARY

Aims: Wilson's disease is an autosomal recessive disorder of copper metabolism due to mutations within *ATP7B* gene. Clinical investigations indicate that ATP7B truncations are associated with an early age of onset when compared to its missense mutations. In vitro studies show that mislocalization of ATP7B mutants is involved in disease-causing mechanisms. Enhanced green fluorescent protein (EGFP) tags are commonly used in *in vitro* studies of cellular localization of ATP7B mutants. However, there is still much unknown about cellular localization of ATP7B truncations. **Methods:** Here, we subcloned full-length human wild-type, a missense mutation (T935M), and four truncating mutants (E332X, Q511X, Q547X, Q819X) of ATP7B into pEGFP-C1, pEGFP-N2 and pCMV-myc, and transfected Chinese hamster ovary (CHO) and SH-SY5Y cells with them, respectively. **Results:** ATP7B truncations all showed a diffuse and homogenous distribution pattern within the cytosol of CHO and SH-SY5Y cells, whereas its wild-type proteins and T935M mutation were clustered in the Golgi apparatus. Furthermore, we found that EGFP tags at N- or C-terminal would severely affect cellular localization of ATP7B truncations, and EGFP tags at N-terminal also have an influence on T935M localization. **Conclusion:** EGFP tags may not be suitable for the detection of cellular localization of ATP7B mutants.

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Introduction

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism due to mutations within *ATP7B* gene [1,2] and clinically characterized by hepatic and neuropsychiatric diseases related to the accumulation of copper in liver and brain [3]. Clinical investigations indicate that ATP7B truncations are associated with a more severe impairment of copper metabolism and an early age of disease onset when compared with missense mutations [4–7]. Therefore, protein-truncating nonsense mutations might have a different impact on the ATP7B function compared with missense mutations.

ATP7B is a membrane-bound, copper-binding protein and disease-causing mutations results in cellular copper overload [8,9]. It is shown that ATP7B is primarily located in the trans-Golgi network, and mislocalization of ATP7B mutants is suggested to be involved in disease-causing mechanisms [10,11]. However, there is still much unknown about cellular localization of ATP7B mutants. GFP/EGFP tags are commonly used in *in vitro* studies of cellular localization and intracellular trafficking of ATP7B mutants [8,10,12], yet some contradictory results were reported. Huster et al. reported that ATP7B-D765N mutant, which was subcloned into pEGFP-C1, exhibited aberrant and clustered localization [12], while D765N, cloned into pCDNA1, was shown predominantly

mislocalized throughout the cell when recognized by immunofluorescence [11].

Here, we subcloned full-length human wild-type, a missense mutation T935M [13], and four truncating mutants including E332X [14], Q511X [15], Q547X [16], and Q819X (W.Ni, Z.Wu, unpublished data) of ATP7B into pEGFP-C1, pEGFP-N2, and pCMV-myc and transfected CHO and SH-SY5Y cells with them, respectively. CHO cells have no endogenous ATP7B expression, whereas SH-SY5Y cells are a human neuroblastoma cell line with endogenous ATP7B expression. The cellular localization of ATP7B proteins was observed by tracking their myc/EGFP tags. To our surprise, we found that EGFP tags at N- or C-terminal would severely affect cellular localization of ATP7B truncations, and EGFP tags at N-terminal also have an influence on T935M localization. The results indicate that EGFP tags may not be suitable for the detection of cellular localization of ATP7B mutants.

Materials and Methods

Cell Culture

SH-SY5Y and CHO cells were obtained from American Tissue Culture Collection and used within 20 passages of the original vial. SH-SY5Y cells were grown in Dulbecco's modified eagle's medium

(DMEM)/F12 medium supplemented with 15% fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 3.7 g/L NaHCO_3 . CHO cells were grown in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 3.7 g/L NaHCO_3 . Cell cultures were all kept at 37°C in a saturated humidity air atmosphere containing 5% CO_2 .

Plasmids and Transfection

Full-length human *ATP7B* cDNA was subcloned into pEGFP-C1, PEGFP-N2, and pCMV-myc expression vectors, respectively. Five mutants of ATP7B (T935M, E332X, Q511X, Q547X, Q819X) were prepared by using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

CHO and SH-SY5Y cells were transfected using X-tremeGENE HP DNA Transfection Reagent (Roche) following the instruction of the manufacturer. Plasmid DNA was first diluted in OPTI-MEM I (Gibco-BRL, Rockville, MD, USA) and then mixed with X-tremeGENE HP DNA Transfection Reagent. Following a 30-min incubation period at room temperature, the mixture mentioned above was added to the antibiotic-free transfection medium (DMEM containing glutamine). The medium was replaced with normal culture medium 6 h later, and the cells were used for the following experiments 48 h after transfection.

The cells over-expressed with EGFP-tagged ATP7B mutants were observed after staining the nucleus with Hoechst (Dojindo, Kumamoto, Japan) on UltraVIEW VoX 3D live cell imaging system (PerkinElmer, Waltham, MA, USA) using a 60× objective, and Z-stacks were taken at 0.5- μm intervals. The cells over-expressed with myc-tagged ATP7B mutants were processed for immunofluorescence as described below before observation.

Golgi-Targeted RFP Transfection

Golgi apparatus was visualized by transfection with a Golgi-targeted RFP kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.

Immunofluorescence

Cells were rinsed with prewarmed PBS (pH 7.2) and fixed in 4% paraformaldehyde for 30 min. After washed with PBS for three times, cells were permeabilized with 0.2% Triton X-100 for 30 min and incubated with 10% fetal calf serum for 90 min. Cells were sequentially incubated with mouse monoclonal anti-myc primary antibody (Abcam) for 2 h and FITC-conjugated anti-mouse IgG (Rockland) for 90 min and washed with PBS between stages. Each step above was carried out at room temperature.

Western Blotting Analyses

Cells were collected after transfection for 48 h and treated with RIPA lysis buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris, pH 8.0, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and protease inhibitors (Roche, EDTA free). The samples were then centrifuged at 12,000 *g* for 20 min at 4°C to remove unsolvable parts. The protein concentration was

determined by a Bio-Rad protein assay (Hercules). Protein samples (10–30 μg) were ran on 10% SDS-PAGE gels. Antibodies used were as follows: rabbit polyclonal anti-ATP7B [17], mouse antibody against β -actin (1:20,000, Sigma), horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Chemicon). Peroxidase activity was visualized with ECL substrate kit (Santa Cruz Biotechnology, Dallas, TX, USA). Images were taken using the Gel Documentation Systems (Bio-Rad, Hercules, CA, USA).

Results

Mislocalization of ATP7B Truncations in Cells

To investigate the cellular localization of ATP7B and its missense mutation, we over-expressed CHO cells with wild-type ATP7B or T935M, both tagged with myc. Then those cells were over-expressed with Golgi-targeted RFP 24 h after the first transfection and processed for immunofluorescence staining of myc after another 24 h. The cellular localization of myc tags and Golgi-targeted RFP was observed on a live cell imaging system. As shown in Figure 1A and Figure 2A, the myc tags (Green) of ATP7B were perfectly overlapped with Golgi-targeted RFP, suggesting that wild-type ATP7B and T935M mutant are normally located in the Golgi apparatus.

Figure 1 Wild-type ATP7B proteins are localized in the Golgi apparatus in CHO cells. CHO cells were first over-expressed with wild-type ATP7B tagged with myc (ATP7B-myc) (A) or EGFP at N-(GFP-ATP7B) (B) or C-terminal (ATP7B-GFP) (C) and then over-expressed with Golgi-targeted RFP 24 h later. The cells over-expressed with myc-tagged ATP7B were processed for immunofluorescence staining of myc 24 h after the second transfection. The cellular localization of myc/EGFP tags (Green) and Golgi-targeted RFP was observed on a live cell imaging system. Representative images show that wild-type ATP7B proteins are localized in the Golgi apparatus in CHO cells, which is not affected by their EGFP tags. Scale bar = 10 μm .

Figure 2 T935M mutation of ATP7B proteins is localized in the Golgi apparatus in CHO cells. CHO cells were over-expressed with T935M mutation tagged with myc (T935M-myc) (A) or EGFP at N- (GFP-T935M) (B) or C- terminal (T935M-GFP) (C) and then over-expressed with Golgi-targeted RFP 24 h later or stained with Hoechst. The cells over-expressed with myc-tagged ATP7B were processed for immunofluorescence staining of myc 24 h after the second transfection. The cellular localization of myc/EGFP tags (Green) was observed on a live cell imaging system. Representative images show that T935M mutation of ATP7B proteins is localized in the Golgi apparatus in CHO cells, which is affected by their EGFP tags at N-terminal. Scale bar = 10 μ m.

Figure 3 Immunoblotting assay of ATP7B in CHO cells over-expressed with four truncating mutants of ATP7B (E332X, Q511X, Q547X, Q819X) and its wild-type protein after transfection for 48 h.

Next, we over-expressed CHO cells with four ATP7B truncations, all tagged with myc, to compare their cellular localization with wild-type ATP7B. Those cells were first processed for immunoblotting assay after transfection for 48 h, and a robust band of full-length or truncating ATP7B was detected in CHO cells (Figure 3). We then compared the cellular localization of ATP7B truncations with its wild-type protein by tracking their myc tags using immunofluorescence staining after transfection for 48 h. As shown in Figure 4, ATP7B wild-type proteins were clustered in Golgi, whereas ATP7B truncations all showed a diffuse and homogenous distribution fashion within the cytosol of CHO cells. We also repeated the experiments in SH-SY5Y cells and found similar results (Figure 5), suggesting a common mislocalization pattern of ATP7B truncations in different types of cells.

EGFP Tags Affect Cellular Localization of ATP7B Mutants

To test the effects of EGFP tags on the cellular localization of ATP7B, we first over-expressed CHO cells with wild-type ATP7B or T935M, both tagged with EGFP at N- or C-terminal. Those cells were then over-expressed with Golgi-targeted RFP 24 h after the first transfection and observed on a live cell imaging system after another 24 h. Both EGFP tags at N- and C-terminal of wild-type ATP7B (Figure 1B,C) were overlapped with Golgi-targeted RFP. However, EGFP tag at N-terminal of T935M (Figure 2B) was mainly located in the nucleus, while EGFP tag at C-terminal of T935M (Figure 2C) was merged with Golgi-targeted RFP.

Then we over-expressed CHO cells with four ATP7B truncations, tagged with EGFP at N- or C-terminal, to test the effects of EGFP tags on the cellular localization of them. The cellular localization of their EGFP tags was observed after transfection for 48 h. As shown in Figure 4, the cellular localization of ATP7B truncations was severely affected by EGFP tags in CHO cells. The EGFP tags at N-terminal of three ATP7B truncating mutants (E332X, Q511X, Q547X) were mostly located in the nucleus, while the EGFP tags at N-terminal of Q819X or at C-terminal of all four mutants spread in the whole CHO cell body in a diffuse distribution fashion. Similar results were also seen in SH-SY5Y cells after over-expressed with ATP7B truncations for 48 h (Figure 5), indicating that the cellular localization of ATP7B truncations was remarkably affected by EGFP tags, and EGFP tags at N- or C-terminal may have different influences on the cellular localization of ATP7B truncations.

Discussion

ATP7B proteins are mainly located in the Golgi apparatus and play an important role in copper transportation; its normal cellular localization is closely connected to its physiological function [10,11]. Here, we not only showed that T935M missense mutation of ATP7B proteins was predominately located in the Golgi apparatus, but also found that four ATP7B truncations all showed a diffuse and homogenous distribution fashion within the plasma of two different kinds of cell lines. It has been reported that Golgi retention signal resides with a 9-amino acid sequence at N-terminal of ATP7B, and its activity needs to be stabilized by other domains [18]. Thus, a possible explanation of our results would be that those four ATP7B truncating mutants are lack of necessary domains to maintain the activity of their Golgi retention signal. ATP7B is known as a membrane-bound protein with eight transmembrane domains, which transports copper across cellular membranes using the energy of ATP hydrolysis [9,14]. Nevertheless, three ATP7B truncating mutants (E332X, Q511X, Q547X) in the current study all lack of transmembrane domains, and Q819X merely contains some of the transmembrane domains. Therefore, it is possible that those ATP7B truncating mutants are just free in the plasma of cells, which still need to be further investigated. The mislocalization of ATP7B truncating mutants probably has an adverse influence on the protein function, which further contributes to the development of WD and leads to a severe impairment of copper metabolism parameters and an early age of disease onset.

Figure 4 Cellular localization of wild-type ATP7B and its truncating mutants in CHO cells. CHO cells were over-expressed with wild-type ATP7B (**A–C**) and its three truncating mutants (E332X (**D–F**), Q511X (**G–I**), Q547X (**J–L**), Q819X (**M–O**)) tagged with myc (ATP7B-myc) or EGFP at N-(GFP-ATP7B) or C-terminal (ATP7B-GFP), respectively. The cells over-expressed with EGFP-tagged ATP7B mutants were observed after staining the nucleus with Hoechst. The cells over-expressed with myc-tagged ATP7B mutants were processed for immunofluorescence before observation. Scale bar = 10 μ m.

Tags of GFP or its modified form EGFP allow analysis of proteins in living cells and have advantages over conventional immunofluorescence, such as lower background, higher resolution, and avoidance of fixation artifacts. Therefore, GFP/EGFP tags are usually used in *in vitro* studies of cellular localization and intracellular trafficking of ATP7B mutants in previous studies [8,10,12]. However, the major disadvantage of studying GFP/EGFP-tagged proteins is that the localization or function of proteins could sometimes be interfered by their tags, regarding to the relatively large molecular weight (~27 kDa) [19,20]. Previous studies also suggest that C-terminal tagging with GFP is generally superior to N-terminal tagging, as tagging with GFP at C-terminal is more likely to preserve the localization of the native protein and therefore to maintain its functional characteristics [21,22]. Similar results were also shown in our present study that the cellular localization of T935M is maintained by C-terminal but not N-terminal tags of EGFP. GFP/EGFP will fold first when tagged at the N-terminal end and possibly disrupt further folding of Golgi retention signal or other domains in ATP7B protein required for its activity and correct localization. This may explain the

contradictory results about cellular localization of ATP7B mutants in previous studies.

Myc tag is also widely used to examine protein localizations, and due to its small size (~2 kDa), they are less likely to disrupt the normal localization or function of proteins [21]. Nonetheless, Myc tag needs to be detected by immunohistochemistry, which is time-consuming and can only be performed on fixed cells [21]. We compared the cellular localization of ATP7B and its four truncating mutants tagged with EGFP or myc. We showed in the present study that the cellular localization of ATP7B truncations but not its wild-type proteins is severely affected by EGFP tags. A possible explanation is that wild-type ATP7B has intact sequences, multiple transmembrane domains, and much larger molecular weight than its truncating mutants, which may help it greatly to be correctly localized. Furthermore, we found here that tagging with EGFP at C-terminal or N-terminal both disrupted the cellular localization of ATP7B truncations. EGFP alone is distributed in the cytoplasm and the nucleus of cells, and it may translocate to the nucleus by diffusion even when fused to a protein, especially of a small molecular weight [20]. That might explain why

Figure 5 Cellular localization of wild-type ATP7B and its truncating mutants in SH-SY5Y cells. SH-SY5Y cells were over-expressed with wild-type ATP7B (A–C) and its three truncating mutants (E332X (D–F), Q511X (G–I), Q547X (J–L), Q819X (M–O)) tagged with myc (ATP7B-myc) or EGFP at N-(GFP-ATP7B) or C- terminal (ATP7B-GFP), respectively. The cells over-expressed with EGFP-tagged ATP7B mutants were observed after staining the nucleus with Hoechst. The cells over-expressed with myc-tagged ATP7B mutants were processed for immunofluorescence before observation. Scale bar = 10 μ m.

EGFP-tagged ATP7B truncations are localized in nucleus. However, EGFP tags at N- or C-terminal have a few different effects on the cellular localization of ATP7B truncations, and the exact reason is unclear.

In summary, we found that ATP7B truncations mislocalized in cells and EGFP tags affected cellular localization of ATP7B mutants including truncating and missense mutants. Our study indicated EGFP tags may not be suitable for studying the cellular localization or intracellular trafficking of ATP7B mutants.

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Conflict of Interest

The authors declare no conflicts of interest.

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