Neuroscience 157 (2008) 588–595

CONSEQUENCES OF THE DYT1 MUTATION ON torsinA OLIGOMERIZATION AND DEGRADATION

K. L. GORDON[a] AND P. GONZALEZ-ALEGRE[a,b,c,d]*

[a]*Interdisciplinary Graduate Program in Neuroscience, Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA*

[b]*Interdisciplinary Graduate Program in Genetics, Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA*

[c]*Interdisciplinary Graduate Program in Molecular and Cellular Biology, Carver College of Medicine, The University of Iowa, Iowa City, IA 52242, USA*

[d]*Department of Neurology, Carver College of Medicine, The University of Iowa, 375 Newton Road, 3111A MERF, Iowa City, IA 52242-1101, USA*

Abstract—DYT1 is the most common inherited dystonia, a neurological syndrome that causes disabling involuntary muscle contractions. This autosomal dominant disease is caused by a glutamic acid deletion near the carboxy-terminus in the protein torsinA. Cell- and animal-based studies have shown how the DYT1 mutation causes mutant torsinA to redistribute from the endoplasmic reticulum to the nuclear envelope, acting through a dominant negative effect over the wild type protein. As a result, the wild type:mutant torsinA expression ratio would be important for disease pathogenesis, and events that influence it, such as a differential degradation process for each protein, might modulate DYT1 pathobiology. The DYT1 mutation also triggers the formation of abnormal intermolecular disulfide bonds in torsinA, although the significance of this finding is unclear. How the protein quality control machinery handles torsinA, and whether this process is affected by its abnormal oligomerization remain unknown. Here, we first explored how the disease-linked mutation influences the catabolic process of human torsinA, demonstrating that the differences in subcellular localization between both forms of torsinA lead to divergences in their degradation pathways and, whereas torsinA is normally recycled through autophagy, the proteasome is also required for the efficient clearance of the mutated form. Subsequently, we determined that the abnormal disulfide bond-dependent oligomerization of mutant torsinA is not a result of its redistribution to the nuclear envelope, but a direct consequence of the mutation. Finally, we established that the presence of disulfide links in mutant torsinA oligomers interfere with their degradation by the proteasome, thus relying on autophagy as the main pathway for clearance. In conclusion, the abnormal subcellular localization and oligomerization of DYT1-linked torsinA influences its catabolic process, opening the door to the modulation of the wild type:mutant torsinA ratio through pharmacological manipulation of protein degradation pathways.

Key words: dystonia, autophagy, proteasome.

Dystonia is a neurological syndrome characterized by disabling twisting involuntary movements (Fahn et al., 1998). While dystonia can be a consequence of different types of brain insults, such as stroke or tumor, several genetic forms of dystonia have been described (Gonzalez-Alegre, 2007). Among those, DYT1 is the most common inherited dystonia. A dominantly inherited disease, DYT1 has limited penetrance with only about a third of mutation carriers developing the clinical syndrome. DYT1 is caused by a GAG deletion in the *TOR1A* gene that causes the loss of a glutamic acid residue in torsinA (torsinA(ΔE)) (Ozelius et al., 1997). The factors that modify DYT1 penetrance remain unknown, although a genetic polymorphism in the disease causing gene plays a small role (Risch et al., 2007).

TorsinA, a widely expressed AAA protein (ATPases Associated with diverse cellular Activities) (Hanson and Whiteheart, 2005), is an endoplasmic reticulum (ER)–resident glycoprotein (Hewett et al., 2000; Kustedjo et al., 2000). The DYT1 mutation does not alter protein solubility (Kustedjo et al., 2003) but causes torsinA to accumulate in the nuclear envelope (NE) (Gonzalez-Alegre and Paulson, 2004; Goodchild and Dauer, 2004; Naismith et al., 2004). When overexpressed in cultured cells, the accumulation of torsinA(ΔE) in the NE triggers the formation of NE-derived cytoplasmic membranous inclusions or spheroid bodies (Gonzalez-Alegre and Paulson, 2004), probably an artifact of overexpression, but a helpful marker of the abnormal behavior of torsinA(ΔE).

Similar to other AAA proteins, torsinA is predicted to assemble into multimers, deriving energy from ATP hydrolysis to mediate conformational changes on substrate proteins (Breakefield et al., 2001). A dominant negative form of torsinA with a mutation that impairs ATP hydrolysis also localizes to the NE (Goodchild and Dauer, 2004; Naismith et al., 2004). Published reports suggest that the presence of torsinA(ΔE) in multimers "locks" them in the NE, acting through a dominant negative effect over torsinA(wt) and leading to a loss of torsinA function (Goodchild and Dauer, 2004; Naismith et al., 2004; Torres et al., 2004; Gonzalez-Alegre et al., 2005). The number of functional multimers, formed exclusively by torsinA(wt), would depend on the torsinA(wt):torsinA(ΔE) expression ratio. Therefore, factors that modulate this ratio, such as a potential differential degradation process for both forms of torsinA, could influence disease pathogenesis and putatively penetrance.

*Correspondence to: P. Gonzalez-Alegre, Department of Neurology, Carver College of Medicine, The University of Iowa, 375 Newton Road, 3111A MERF, Iowa City, IA 52242-1101, USA. Tel: +1-319-335-9178; fax: +1-319-335-9517.
E-mail address: pedro-gonzalez-alegre@uiowa.edu (P. Gonzalez-Alegre).

Abbreviations: AAA, ATPases Associated with diverse cellular Activities; DOX, doxycycline; DTT, dithiothreitol; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated degradation; IF, indirect immunofluorescence; NE, nuclear envelope; NEM, N-ethylmaleimide; 3MA, 3-methyladenine.

0306-4522/08 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.09.028

How neurons handle abnormal proteins is critical in the pathogenesis of many neurological diseases. Whereas the degradation of NE-resident proteins has not been investigated, ER glycoproteins are usually degraded by the proteasome through ERAD (endoplasmic reticulum–associated degradation) (Meusser et al., 2005; Romisch, 2005) or by the lysosome through macroautophagy (Cuervo, 2004; Kruse et al., 2006) (referred to as autophagy from now on). In ERAD, ER proteins that retain a high mannose tag are selectively retrotranslocated to the cytoplasm for proteasomal degradation. In autophagy, cells recycle macromolecules and organelles such as the ER non-selectively by engulfing them in a double-membrane structure that fuses to the lysosome. As a high-mannose glycoprotein that resides within the secretory pathway, torsinA is a potential a substrate for both systems. However, the different subcellular localization of torsinA(wt) and torsinA(ΔE) (ER versus NE, respectively), could lead to divergences in their catabolic process.

Another differential feature of torsinA(Δ E) is that it forms abnormal intermolecular disulfide links (Gonzalez-Alegre and Paulson, 2004; Torres et al., 2004). Whether this abnormal oligomerization is responsible for the functional defects caused by torsinA(ΔE) is unknown, but a recent report identified torsinA as a redox sensor with critical *intra*molecular disulfide bonds that regulate its nucleotide binding activity (Zhu et al., 2008), demonstrating a potential role for this abnormal molecular event in DYT1 dysfunction. Furthermore, because reduction of disulfide links is a required step prior to retrotranslocation for proteasomal degradation (Romisch, 2005), this abnormal feature of torsinA(ΔE) could also influence its degradative process and, as explained above, modulate DYT1 pathogenesis.

Here, we completed experiments to determine whether differences in subcellular localization and disulfide-link dependent oligomerization between torsinA(wt) and torsinA(ΔE) lead to divergences in their catabolic process, which could implicate the protein quality control machinery in DYT1 pathogenesis.

EXPERIMENTAL PROCEDURES

Cell culture, transfection, non-reducing lysis and Western blot

Cos7 cells were grown, maintained and transiently transfected using LIPOfectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described (Gonzalez-Alegre et al., 2003). TorsinA(wt) and torsinA(ΔE) inducible PC6-3 clonal cell lines were grown, differentiated on nerve growth factor, maintained and induced as previously reported (Gonzalez-Alegre and Paulson, 2004). Differentiation with nerve growth factor and 1% HS, 0.5% FBS (both from Gibco Grand Island, NY, USA) low serum media were used to avoid cell division and achieve a differentiated neural phenotype. To induce transcription of the transgene we employed doxycycline (DOX) at 1.5 μg/mL unless otherwise noted. SH-SY5Y cells were grown in DMEM/F12 with 10% fetal bovine serum, 1.0 mM Na pyruvate and 0.1 mM NEAA (all from Gibco). HEK293 cells were grown in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco). To obtain protein lysates in reducing conditions, cells were harvested at the indicated times using Laemmli buffer with 200 mM dithiothreitol (DTT; Research Products International, Mt. Prospect, IL, USA), boiled and sonicated as described (Gonzalez-Alegre and Paulson, 2004). For non-reducing conditions, cells were harvested using Laemmli buffer without DTT, adding 10 mM N-ethylmaleimide (NEM; Sigma, St. Louis, MO, USA) to avoid aberrant disulfide link formation, boiled and sonicated. An aliquot from each non-reduced lysate was placed into a different Eppendorf, and reduced by addition of 200 mM DTT (Gonzalez-Alegre and Paulson, 2004). Samples were run using SDS-PAGE, transferred to a PVDF membrane and Western blot completed as described (Gonzalez-Alegre et al., 2003).

Quantification of Western blot signal

To quantify Western blot band intensity, signal was determined and normalized to loading control as previously described (Gonzalez-Alegre et al., 2003, 2005). In brief, blots were scanned, and the pixel intensity of the bands was quantified using the Scion Image software (Scion, Frederick, MD, USA). TorsinA signal was normalized to the loading control and the result expressed as a percentage of the signal obtained in the control lane.

Plasmid vectors

Expression vectors encoding human torsinA(wt) and torsinA(ΔE) were previously described (Gonzalez-Alegre et al., 2003). Vectors encoding human torsinA carrying the Walker A (K108A) and Walker B (E171Q) mutations were a generous gift of P. Hanson (Washington University, St. Louis, MO, USA) and W. Dauer (Columbia University, New York, NY, USA) (Goodchild and Dauer, 2004; Naismith et al., 2004). Plasmid vectors encoding mouse torsinA carrying an internal myc tag and human torsinA lacking the hydrophobic stretch that follows the signal peptide (torsinA(wt)(Δ24-40)), were a kind gift of Drs. W. Dauer and M. Zolkiewski, respectively.

Antibody generation

TorsinA antibodies were generated by Sigma Genosys (The Woodlands, TX, USA) by inoculating New Zealand white rabbits with a keyhole limpet hemocyanin-conjugated peptide containing the last 14 amino acids of human torsinA. For affinity purification, immune serum was incubated overnight at 4 °C with the same peptide conjugated to agarose beads, which were then pelleted and washed with TBS (25 mM Tris, pH 7.4, 2.7 mM KCl, 14 mM NaCl). The purified antibody was eluted with four successive bead volumes of glycine, pH 2, and aliquots containing antibody were pooled. TorsinA antibodies (TA913), pre-immune and pre-adsorbed serum were diluted in 5% nonfat dry milk, and antibody specificity was confirmed with Western analysis (supplemental Fig. 1). Other antibodies employed included FLAG M5 (Sigma), α-tubulin (Sigma), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), DM2A8 antibody against torA (Cell Signaling Technology, Boston, MA, USA) and ubiquitin (DakoCytomation, Carpenteria, CA, USA).

Indirect immunofluorescence (IF)

Cells were seeded in four-chamber slides and transfected, or induced by treatment with DOX, and processed as previously described unless otherwise noted (Gonzalez-Alegre et al., 2003, 2005; Gonzalez-Alegre and Paulson, 2004). In brief, cells were fixed in 4% paraformaldehyde at room temperature for 15 min, incubated sequentially with washes in the indicated primary and corresponding secondary antibodies, nuclei stained with DAPI and slides mounted and visualized with a Zeiss (Thornwood, NY, USA) Axioplan fluorescence microscope or an Olympus (Center Valley, PA, USA) BX-51 digital light microscope. Digital images were collected on red, green and blue fluorescence channels using an AxiocamHRm (Zeiss) digital camera or a Diagnostics Instruments (Sterling Heights, MI, USA) SPOT digital camera. Secondary antibodies used were Alexa Fluor® 568– (Invitrogen), FITC- or Rhodamine-conjugated antibodies (Santa Cruz Biotechnology).