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MUTANT TORSINA INTERACTS WITH TYROSINE HYDROXYLASE IN CULTURED CELLS

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Abstract—A specific mutation (ΔE302/303) in the torsinA gene underlies most cases of dominantly inherited early-onset torsion dystonia. This mutation causes the protein to aggregate and form intracellular inclusion bodies in cultured cells and animal models. Co-expression of the wildtype and mutant proteins resulted in the redistribution of the wildtype protein from the endoplasmic reticulum to inclusion bodies in cultured HEK293 cells, and this was associated with increased interaction between the two proteins. Expression of ΔE302/303 but not wildtype torsinA in primary postnatal midbrain neurons resulted in the formation of intracellular inclusion bodies, predominantly in dopaminergic neurons. Tyrosine hydroxylase was sequestered in these inclusions and this process was mediated by increased protein–protein interaction between mutant torsinA and tyrosine hydroxylase. Analysis in an inducible neuroblastoma cell culture model demonstrated altered tyrosine hydroxylase activity in the presence of the mutant but not wildtype torsinA protein. Our results suggest that the interaction of tyrosine hydroxylase and mutant torsinA may contribute to the phenotype and reported dopaminergic dysfunction in torsinA-mediated dystonia. **© 2009 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Primary dystonias are a heterogeneous group of neurologic conditions characterized by involuntary, sustained muscle contractions affecting one or more body segments, with dystonia as the sole or major symptom (Nemeth, 2002). Early-onset torsion dystonia (EOTD) is the most common and severe form of dystonia and is often associated with mutation of the DYT1 gene torsinA. In torsinA dystonia, deletion of one of a pair of glutamate residues (ΔE302/303) in the carboxy-terminal region of torsinA is associated with autosomal dominant inheritance with reduced penetrance (Ozelius et al., 1997). The neuropathology of torsinA dystonia has been described in several cases and the apparent absence of neuronal loss has led to the suggestion that torsinA dystonia is not a degenerative disease but is primarily due to neuronal dysfunction (Walker et al., 2002; Rostasy et al., 2003; McNaught et al., 2004).

The exact function of torsinA is unknown, but homology to AAA+ ATPases (Neuwald et al., 1999; Ogura and Wilkinson, 2001) and functional studies suggest a possible chaperone role (McLean et al., 2002; Caldwell et al., 2003; Cao et al., 2005). AAA+ proteins often self-associate and function as an oligomeric complex and torsinA has previously been shown to form higher molecular weight species and oligomerize (Kustedjo et al., 2000; Torres et al., 2004). In addition, torsinA has been shown to interact with a number of proteins of diverse function. This includes cytoskeletal-associated cytoplasmic proteins such as kinesin light chain (KLC), vimentin and actin (Kamm et al., 2004; Hewett et al., 2006). TorsinA also interacts with nuclear envelope (NE) and endoplasmic reticulum (ER) resident proteins LAP1, LULL1, nesprin-3 and printor (Goodchild and Dauer, 2005; Nery et al., 2008; Giles et al., 2009). The identification of multiple binding partners suggests that disruption of torsinA interactions might contribute to the disease phenotype. The ability to correctly oligomerize has been shown to be necessary for AAA+ ATPase function in several proteins (reviewed in; Mogk et al., 2008). Moreover, the ΔE302/303 deletion has been demonstrated to increase interaction of the mutant protein with both itself and nesprin-3 (Torres et al., 2004; Nery et al., 2008). A feature of the ΔE302/303 mutation is the aggregation and mislocalization of the protein to perinuclear inclusion bodies and the nuclear envelope in cell and animal models (Hewett et al., 2000; Goodchild and Dauer, 2004; Goodchild et al., 2005), suggesting a potential dominant-negative disease mechanism involving sequestration of both wildtype and interacting proteins (Torres et al., 2004).

There is considerable evidence implicating an imbalance in dopaminergic function in the aetiology of dystonia. Individuals with DYT1 dystonia demonstrated an increase in the turnover of dopamine with reduced D1 and D2 receptor binding in the striatum, suggestive of an imbalance in dopamine signaling (Augood et al., 2002; Asanuma et al., 2005). In addition, nigral dopaminergic neurons tended to be larger in size in DYT1 patients when compared to controls (Rostasy et al., 2003) and ubiquitin-positive aggregates have been identified in pigmented dopa-

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Abbreviations: AADC, aromatic acid decarboxylase; ER, endoplasmic reticulum; TH, tyrosine hydroxylase; Tyr–AMC, tyrosine-7–amino-4-methylcoumarin.

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minergic neurons of the substantia nigra pars compacta and locus ceruleus (McNaught et al., 2004). Similarly, cell and animal models have been utilized to investigate the involvement of the dopaminergic system in DYT1 dystonia and improve our understanding of disease pathophysiology. Transgenic mice over expressing human mutant torsinA have been reported to display alterations in striatal dopamine transport and/or release and turnover (Balcioglu et al., 2007; Zhao et al., 2008). Moreover, *in vitro* studies have also identified a role for torsinA in both dopamine transporter function (Torres et al., 2004; Cao et al., 2005) and more generally in synaptic vesicle recycling through an interaction with snapin (Granata et al., 2008). To test for the direct involvement of torsinA with the dopaminergic system, we examined whether wildtype or mutant torsinA interacted with components of the dopamine synthesis pathway, focusing on tyrosine hydroxylase (TH), the rate limiting step in dopamine synthesis.

EXPERIMENTAL PROCEDURES

Plasmids, antibodies and reagents

Amplification of cDNA for wildtype torsinA and mutagenesis to produce deletion mutants have been described elsewhere (O'Farrell et al., 2002). C-terminal V5 or myc tagged versions of the same constructs were generated by subcloning into pCDNA3.1V5His and pCDNA3.1Hismyc respectively. TorsinB was amplified from adult human brain cDNA using the Advantage PCR kit (both from Clontech, Mountain View, CA, USA), using the following primers (Forward 5′-CGAGGAGCGGGATGTTGCGG-3′ Reverse 5′-TCCGTGGAAATCCAGCCGCGA−3) and likewise cloned into pCDNA3.1/V5-his TOPO using the TA cloning system (Invitrogen, Carlsbad, CA, USA). Primary antibodies utilized included polyclonal anti-torsinA (1:1000, torsin290; O'Farrell et al., 2002); monoclonal anti-V5 (1:5000, R960-25, Invitrogen, Carlsbad, CA, USA); polyclonal anti-TH (1:2000, AB1542, Chemicon, IL, USA); polyclonal anti-V5 (1:400, AB3792, Chemicon, IL, USA); monoclonal anti-myc (1:1000, #2276, Cell Signaling, Danvers, MA, USA), monoclonal anti–PDI (1:50, SPA-891, Stressgen, San Diego, CA, USA), monoclonal anti-map 2 (1:500, M1406, Sigma, Dorset, UK), polyclonal anti-dopamine β hydroxylase (1:1500, AB1585, Millipore, Herts, UK), monoclonal anti-β-actin (1:2000, A5441, Sigma, Dorset, UK), monoclonal anti-nucleoporin (1:2000, 610498, BD Transduction Laboratories, Lexington, KY, USA) and monoclonal anti-DOPA decarboxylase (1:1000, D0180, Sigma, Dorset, UK).

Cell culture

Human embryonic kidney cells (HEK-293T) and human neuroblastoma BE(2)–M17 cells were cultured in Opti–MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/mL). Cells were plated 24 h prior to transient transfection in six-well culture plates at 2×10^5 cells per well and transfected with the various constructs using Fugene6 (Roche, Mannheim, GmbH) according to the manufacturer's protocols. An SY5Y Tet-on cell line maintained in 400 μg/mL G418 was stably transfected with pTRE2hyg constructs containing either wildtype torsinA, ΔE302/303 torsinA or an unrelated control protein (PACRG) using Fugene6. Clonal cell lines stably expressing torsinA (isolated by limiting dilution) were maintained in 50 μg/mL hygromycin. Cell lines were induced with the addition of 2 μg/mL doxycycline daily for 4 days prior to harvesting or assaying. Primary cell cultures were prepared from post-natal mouse midbrain using methods described previously (Mena et al., 1997; Burke et al., 1998; Petrucelli et al., 2002). Briefly, midbrains containing substantia nigra (SN) and ventral tegmental area were dissected from 2-day-old postnatal mouse pups using anatomical landmarks as described. Neurons from these areas were dissociated with papain and plated on top of pre-established cortical glia cell monolayers. For viral transductions, wildtype or mutant torsinA cDNAs were subcloned into the pHSVPrpUC amplicon, packaged into recombinant viral particles using 5dl1.2 helper virus and the 2–2 packaging cell line (Neve et al., 1997) and purified using sucrose gradients and titres determined as described previously (Petrucelli et al., 2002). Cells were transduced with HSV vectors for wildtype or mutant torsinA at a multiplicity of infection (MOI) of 10 and analysed 48 h later.

Western blot and co-immunoprecipitation analysis

Cells were harvested in extraction buffer containing 10 mM Tris–HCl, pH 7.5, 2% SDS and protease inhibitors (P8340, Sigma, Dorset, UK) and protein was estimated by the BCA method (Pierce, IL, USA). Lysates (10 μg total protein per lane) were separated on 10% SDS–PAGE gels and transferred to PVDF membranes (Immobilon, Millipore, Herts, UK). Membranes were incubated in blocking buffer (5% skim milk in TBS–Tween) for 1 h at room temperature. Primary antibodies were allowed to incubate overnight at 4 °C. Antibody binding was revealed using peroxidase conjugated secondary antibodies donkey anti-rabbit (1:10,000 dilution, 711-035-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and donkey anti-mouse (1:10,000 dilution, 715-035-150, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and enhanced chemiluminescence (ECL, Amersham, GE Healthcare, UK) according to the manufacturer's protocols. Signals were exposed to film or quantitation of protein expression was performed by capturing ECL using a CCD-camera based system (Alphalmager, Alpha Innotech Corp., San Leandro, CA, USA). For immunoprecipitation experiments, transfected cells were scraped in cold PBS and collected by centrifugation then resuspended by briefly sonicating in buffer containing 150 mM NaCl, 50 mM TRIS–HCl (pH 8.0) 0.1% v/v TritonX100, 1 mM PMSF and protease inhibitor cocktail (P8340, Sigma, Dorset, UK). Lysates were pre-cleared with immobilized proteinG (20398, Pierce, IL, USA), immunoprecipitated with primary antibody overnight at 4 °C and captured with proteinG-agarose beads. After washing five times in immunoprecipitation buffer, protein was released from beads by heating in the presence of Laemmli sample buffer and immunoprecipitated complexes were analysed by Western blotting as above.

Immunofluorescence

Cells were fixed 48 h after transfection by immersion in methanol at −20 °C for 10 min, washed with PBS and non-specific immunoreactivity was blocked with PBS containing 10% FBS and 0.1% Tween-20. Primary antibodies were diluted in PBS with 1% BSA (wt/vol), applied and allowed to incubate overnight at 4 °C. Cells were then washed twice in PBS for 5 min and incubated with secondary antibodies (1:1000 goat anti-rabbit AlexaFluor 488, A11034 and goat anti-mouse AlexaFluor 594, A11032, Molecular probes, Invitrogen, Carlsbad, CA, USA) for 1–2 h at room temperature. Cells were again washed twice for 5 min before mounting under ProLong antifade medium (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Primary cells were triple stained using sheep anti-TH, mouse anti-MAP2 and rabbit anti-torsinA. In some experiments we also used a monoclonal antibody to the ER marker PDI in place of MAP2. Omission of primary antibody was used to evaluate non-specific fluorescence and in all cases gave no signal. For counting of torsinA inclusions in primary neurons, the operator was blinded to the torsinA construct used (mutant vs. WT) and to the neuronal cell type ($TH^{\pm}$) when counts were made. Following assessment for the presence of torsinA inclusions, neurons were subsequently assigned to TH^+ and TH^- groups. Data shown represent at least two separate experiments where the

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