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TARDBP pathogenic mutations increase cytoplasmic translocation of TDP-43 and cause reduction of endoplasmic reticulum Ca^{2+} signaling in motor neurons

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ABSTRACT

The transactive response DNA binding protein (TDP-43) is a major component of the characteristic neuronal cytoplasmic inclusions seen in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Furthermore, pathogenic mutations in the gene encoding TDP-43, *TARDBP*, are found in sporadic and familial ALS cases. To study the molecular mechanisms of cellular toxicity due to TDP-43 mutations we generated a novel *in vitro* cellular model using a fluorescently tagged human genomic *TARDBP* locus carrying one of two ALS-associated mutations, A382T or M337V, which were used to generate site-specific bacterial artificial chromosome (BAC) human stable cell lines and BAC transgenic mice. In cell lines and primary motor neurons in culture, TDP-M337V mislocalized to the cytoplasm more frequently than wild-type TDP (wt-TDP) and TDP-A382T, an effect potentiated by oxidative stress. Expression of mutant TDP-M337V correlated with increased apoptosis detected by cleaved caspase-3 staining. Cells expressing mislocalized TDP-M337V spontaneously developed cytoplasmic aggregates, while for TDP-A382T aggregates were only revealed after endoplasmic reticulum (ER) stress induced by the calcium-modifying drug thapsigargin. Lowering Ca^{2+} concentration in the ER of wt-TDP cells partially recapitulated the effect of pathogenic mutations by increasing TDP-43 cytoplasmic mislocalization, suggesting Ca^{2+} dysregulation as a potential mediator of pathology through alterations in Bcl-2 protein levels. Ca^{2+} signaling from the ER was impaired in immortalized cells and primary neurons carrying TDP-43 mutations, with a 50% reduction in the levels of luminal ER Ca^{2+} stores content and delayed Ca^{2+} release compared with cells carrying wt-TDP. The deficits in Ca^{2+} release in human cells correlated with the upregulation of Bcl-2 and siRNA-mediated knockdown of Bcl-2 restored the amplitude of Ca^{2+} oscillations in TDP-M337V cells. These results suggest that TDP-43 pathogenic mutations elicit cytoplasmic mislocalization of TDP-43 and Bcl-2 mediated ER Ca^{2+} signaling dysregulation.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease of adult onset, and is characterized by combined loss of upper and lower motor neurons. Between 5 and 10% of cases of ALS show an autosomal dominant pattern of inheritance (familial ALS, fALS), but genetic factors are relevant to the etiology of a significant proportion of cases of sporadic ALS (sALS) (Al-Chalabi et al, 2012). The pathological hallmark of ALS is the presence of ubiquitinated neuronal cytoplasmic inclusions (Leigh et al, 1991). The transactive response DNA binding protein (TDP-43) was identified as a major component of neuronal cytoplasmic inclusions in the overwhelming majority of ALS cases and in a significant subgroup of the related neurodegenerative disorder frontotemporal lobar degeneration (FTLD) (Neumann et al, 2006), suggesting a pathogenic link between these disorders and leading to their re-classification as 'TDP-43 proteinopathies' (Cohen et al, 2011). The formation of granular cytoplasmic aggregates, seen at various stages of pathology in ALS and FTD, are postulated to represent 'pre-inclusions', and therefore are a legitimate focus for *in vitro* models of the early events in ALS pathogenesis. Inclusions with a variety of

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other morphologies, including nuclear aggregates, are seen in ALS and FTLD, but their significance is uncertain.

TDP-43, encoded by the gene *TARDBP*, is a ubiquitously expressed nuclear protein with an important role in the regulation of gene expression by modulating transcription, splicing, stability of mRNA, as well as microRNA biogenesis (Buratti et al, 2001; Mercado et al, 2005; Ou et al, 1995; Strong et al, 2007). Deletion of *TARDBP* leads to embryonic lethality and in neuronal cultures loss of TDP-43 induces pre-mRNA missplicing and neuronal dysfunction (Daoud et al, 2009; Kabashi et al, 2008; Kraemer et al, 2010; Wu et al, 2010). Approximately 30 different mutations have been identified in *TARDBP*, accounting for approximately 4% of fALS patients and a small minority of cases of sALS, suggesting that it has a direct role in the pathogenesis of ALS (Daoud et al, 2009; Gitcho et al, 2008; Sreedharan et al, 2008; Van Deerlin et al, 2008; Yokoseki et al, 2008). The majority of mutations are concentrated in the C-terminal glycine-rich domain of TDP-43 and are predicted to result in abnormal RNA–protein or protein–protein interactions (Pesiridis et al, 2009).

There is evidence that a combination of Ca^{2+} influx during neurotransmission and low cytosolic Ca^{2+} buffering contributes to the vulnerability of motor neurons in ALS, with disordered intracellular calcium homeostasis promoting early protein aggregation into inclusions (Tradewell et al, 2011; Tradewell and Durham, 2010). A recent study using genome-wide RNAi screening to identify regulators of the nucleocytoplasmic shuttling of TDP-43 has indicated that TDP-43 expression and localization are under the control of Ca^{2+} and reduction in endoplasmic reticulum (ER) Ca^{2+} flux promotes nuclear exclusion and degradation of TDP-43 (Kim et al, 2012).

The ER plays a vital role in many cellular processes including Ca^{2+} storage and protein folding. ER stress markers are upregulated in ALS motor neurons at autopsy and in *in vitro* cell models (Atkin et al, 2008; Ilieva et al, 2007). TDP-43 is known to regulate expression and splicing of the proto-oncogene Bcl-2, a candidate for regulating ER Ca^{2+} by binding to the IP_3 receptor and inhibiting the channel's opening to release Ca^{2+} into the cytosol, thereby altering Ca^{2+} signaling from the ER (Sephton et al, 2011). The Bcl-2 family of proteins is a group of evolutionarily conserved regulators of cell death and encompasses both anti-and pro-apoptotic members, operating on the mitochondrial membrane or ER to regulate the permeability of membranes. Bcl-2 has been shown to increase ER Ca^{2+} leakage through phosphorylation of the IP_3R resulting in a decrease in the concentration of free Ca^{2+} within the ER (Foyouzi-Youssefi et al, 2000; Sharpe et al, 2004).

To study the physiological effects of disease-associated TDP-43 mutations, we developed a novel TDP-43 cellular model using the full-length genomic *TARDBP* locus engineered to express either wild-type TDP-43 (wt-TDP), or the A382T or M337V mutations, tagged with the green fluorescent protein (GFP) variant, Ypet. The TDP-M337V mutant showed increased cytoplasmic mislocalization compared with wt-TDP and TDP-A382T, and this was potentiated by oxidative stress in HEK293 clonal cell lines. Expression of TDP-M337V correlated with high levels of cleaved caspase-3. Calcium signaling studies in HEK293 cell lines expressing TDP-43 variants revealed delayed, low amplitude Ca^{2+} spikes in cells expressing TDP-M337V and TDP-A382T compared with untransfected and wt-TDP upon carbachol stimulation of ER Ca^{2+} release. Furthermore, decreased Ca^{2+} levels were detected in the ER of human cells expressing TDP-M337V and TDP-A382T, and also in spinal motor neurons from transgenic mice carrying the TDP-M337V human genomic construct. These phenotypes were associated with increased levels of Bcl-2. Knockdown of Bcl-2 increased the amplitude of Ca^{2+} oscillations in TDP-M337V cells to levels similar to those seen with wt-TDP, suggesting that TDP-43 might be involved in calcium regulation and ALS-associated TDP-43 mutations may contribute to altered ER Ca^{2+} homeostasis through altered Bcl-2 regulation.

Materials and methods

Full-length genomic TDP-43 vector construction and transfections

We obtained the pBACe3.6 plasmid RP11-829B14 containing *TARDBP* full-length genomic locus of 202.43 kb from the BACPAC Resources Centre at Children's Hospital Oakland Research Institute, Oakland, CA. The 47.5 kb *TARDBP* genomic locus, including a 30 kb regulatory upstream region and a 5 kb downstream region, was subcloned into a 9.5 kb pCYPAC2 plasmid using a Red/ET-based strategy for homologous recombination, as previously described (Alegre-Abarrategui et al, 2009). Briefly, the pCYPAC2 plasmid was amplified with 4 primers for the addition of 110 bp homology arms to the upstream and downstream regions of interest in the *TARDBP* genomic locus, and correct subcloning was assessed by restriction digests, and PCR. The Ypet reporter gene was inserted by two rounds of homologous recombination, which involved a two-step process based on the dual resistance/sensitivity cassette pRpsl-Chl. First, the pRpsl-Chl plasmid was amplified with homology sequences of 110 bp to the immediate downstream region of *TARDBP* and inserted by homologous recombination. Next, the Ypet sequence was amplified with homology sequences to the same region and a second round of homologous recombination was performed, exchanging the pRpsl-Chl cassette with Ypet.

Conventional site-directed mutagenesis was used to insert A382T and M337V missense mutations in exon 6 using Stratagene Pfu Turbo polymerase. The mutations were each engineered in separate plasmids carrying the cDNA of *TARDBP* (A382T exchange bases G->A, M337V exchange bases A->G) and the entire exon 6 carrying the correct mutations was exchanged with exon 6 from TDP-43 PAC. The vector wt-TDP-FRT, which was used for developing HEK293 clonal cell lines, was obtained by retrofitting 1 μg of wt-TDP-PAC with 3 ng of pH-FRT-Hy using Cre recombinase according to the manufacturer's instructions. The plasmids were transiently transfected into HEK293 cells by Lipofectamine/Plus following the manufacturer's instructions (Invitrogen). The cells were lysed 4 days following transfection for protein analysis.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and then scraped into PBS. Cell were lysed in RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40 and 1% protease inhibitor cocktail (Sigma)), sonicated and incubated on ice for 30 min. After centrifugation at 3000 *g* for 10 min, the supernatant was retained and protein concentration was quantified using the BCA assay according to the manufacturer's instructions (Sigma). Protein was boiled in Laemmli buffer for 5 min at 95 °C and 5–10 μg were loaded on gels. For Western blot analysis, lysates were resolved on a SDS-PAGE (10% or 12% Tris–glycine gel) using a Mini-Protean Tetra electrophoresis system (BioRad) under constant current (120 mA for 1 h) and transferred to a methanol activated polyvinylidene difluoride membrane (Immobilon-P, PVDF) using the Mini Trans-blot cell system under constant voltage (100 V for 70 min). Blots were blocked in a blocking buffer (TBS, 1% Tween-20, 5% skimmed milk) for 1 h at room temperature and then incubated in TBS + 1% Tween-20 + 1% milk with primary antibodies overnight at 4°C. Primary antibodies used were rabbit or mouseanti-TARDBP (ProteinTech, 1:500), rabbitanti-GFP (Life Technologies, 1:1000), mouseanti-Bcl-2 (Dako, 1:200), mouseanti-p62 (BD Biosciences, 1:1000), rabbitanti-GRP78/BiP (Abcam, 1:1000), rabbitanti-β-actin (Abcam, 1:1000), mouseanti-GAPDH (Abcam, 1:1000). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (BioRad) was used as secondary antibody and the signal was visualized using an ECL or ECL Plus detection system (Amersham Pharmacia Biotech). The integrated optical density of each band was measured in ImageJ and expression was normalized to actin levels in the same blot for comparative expression assessment.