A Direct Interaction between Causative Genes of DYT1 and DYT6 Primary Dystonia

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Abstract

Primary dystonia is a movement disorder characterized by sustained muscle contractions and in which dystonia is the only or predominant clinical feature. *TOR1A* (*DYT1*) and the transcription factor *THAP1* (*DYT6*) are the only genes identified thus far for primary dystonia. Using electromobility shift assays and chromatin immunoprecipitation (ChIP)-qPCR, we demonstrate a physical interaction between THAP1 and the *TOR1A* promoter that is abolished by pathophysiologic mutations. Our findings provide the first evidence that causative genes for primary dystonia intersect in a common pathway and raise the possibility of developing novel therapies targeting this pathway.

Introduction

Mutations in the transcription factor *THAP1* were recently found to cause dystonia type 6 [1-7], Many of the mutations that have been identified are located within the THAP domain, a sequence-specific DNA-binding domain belonging to the zinc-finger superfamily [8-11]. Our previous study showed that a single point mutation (F81L) in the DNA binding domain of THAP1 impaired its DNA binding affinity [1], suggesting that THAP1 mutations may cause a transcriptional dysregulation of THAP1 downstream targets.

THAP1 has been shown to be a modulator of Ribonucleoside-diphosphate reductase large subunit 1 (*RRM1*) and several other pRB/E2F cell-cycle target genes in endothelial cells, including mitotic arrest deficient 2 (*MAD2*), survivin (*BIRC5*), hyaluronan-mediated motility receptor (*HMMR*), Ribonucleoside-diphosphate reductase large subunit 2 (*RRM2*), cell division cycle 2 (*CDC2*), cyclin B1, and Disks Large Homolog 7 (*DLG7*) [12], but these genes have not been linked to dystonia. As clinically defined forms of primary dystonia have been mapped to distinct loci with no obvious physiological link, no common pathways have

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emerged. However, the phenotypic overlap among forms of primary dystonia suggests that THAP1 could represent a link *via* transcriptional regulation of other dystonia genes. We therefore investigated whether the *TOR1A* gene mutated in dystonia type 1, might be a transcriptional target gene for THAP1. In the present study we demonstrated that *TOR1A* is a direct target of endogenous THAP1 in human primary cells and in mouse brain.

Results

A previous study 9 has determined that THAP1 binds to an 11-bp consensus DNA sequence, the THAP-domain-binding sequence (THABS). Sequence evaluation of the human *TOR1A* minimal gene promoter 13 for putative THABS predicted an inverted THABS at nucleotides -111/-101 relative to the translation initiation site of the *TOR1A* gene: c**TGCC**ctg**A**ag. To determine whether THAP1 binds to the *TOR1A* promoter, nuclear extracts from 293T cells transfected with wild type human THAP1 were subjected to electrophoretic mobility shift assays using the human *TOR1A* fragment -159/-80 as a probe. We detected two protein/ DNA complexes that were specific to THAP1 over-expression and were competed off by an excess of unlabelled probe (Fig 1A), indicating that these interactions were specific. The presence of THAP1 in the complexes was confirmed by their supershift with an antibody to THAP1 (Fig 1A). The GGCA core motif of the THABS was previously shown to be required for THAP-domain–THABS interaction 9. Consistently, EMSA with the *TOR1A* probe -159/-80 mutated in its core consensus site (AGCA probe) did not reveal a shift with the same cellular extract (Fig 1B).

Next, to assess the association of THAP1 with the *TOR1A* promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP)-qPCR assays in human primary cells (human umbilical vein endothelial cell, HUVECs) and the T98G glioblastoma cell line. In both cell types, THAP1 bound to the endogenous *TOR1A* promoter, but not to a control genomic site (*RRM1* Exon 19) that does not contain a THABS motif (Fig 1C). Consistent with the specificity of our ChIP assays, the control antibodies did not immunoprecipitate significant levels of either DNA sequence. Quantification indicated an approximate 26- and 21-fold enrichment of THAP1 on the *TOR1A* promoter (compared to the control genomic site) in HUVECs and T98G cells, respectively (Fig 1D). ChIP-qPCR assays in cells treated with THAP1 siRNAs revealed a significant reduction of THAP1 association with the *TOR1A* promoter, consistent with reduced levels of THAP1 protein (see Supplementary Fig 1). Taken together with the EMSA DNA-binding assays, these *in vivo* ChIP assays indicate that *TOR1A* is a direct target of endogenous THAP1.

Physiological interaction between THAP1 and the *TOR1A* gene was further investigated *in vivo* in mouse brain tissue. Immunoblot analysis revealed that THAP1 concentration was highest in embryonic whole brain tissue and declined after birth in compared brain regions (Fig 2A). This pattern is similar to what has been reported for torsinA 14, 15, the protein encoded by *TOR1A*. In addition, ChIP-qPCR assays with adult mouse brain chromatin revealed robust binding of endogenous THAP1 to the *Tor1A* and *Rrm1* (positive control) promoters (> 3% of total input DNA precipitated with THAP1 antibodies), whereas binding of THAP1 to a control genomic site (*Tor1A exon 5*) was similar to the background levels obtained with control antibodies (Fig 2B). Quantification indicated a > 65-fold enrichment of THAP1 on the *Tor1A* and *Rrm1* promoters in mouse brain (Fig 2C). Importantly, very similar results were obtained with two independent THAP1 antisera and two distinct control antibodies (Fig 2B and 2C). We concluded that THAP1 is expressed in the brain and binds to the *Tor1A* promoter *in vivo*.

We further hypothesized that disruption of the THAP1/*TOR1A* interaction may be involved in the molecular pathogenesis of dystonia type 6. To examine this hypothesis, we carried out

EMSA experiments using nuclear extracts of 293T cells transfected with the previously characterized F81L THAP1 mutant, as well as two other pathophysiologic THAP1 missense mutants, C54Y (Fuchs T. *et al.* in preparation) and S21T 2 all of which are located within the THAP domain. Immunoblots from these nuclear extracts verified that the THAP1 mutants were efficiently expressed (Fig 3A). EMSA studies with these extracts showed that each of the three distinct missense mutations abolish binding of THAP1 to *TOR1A* (Fig 3B). Furthermore, ChIP-qPCR assays using T98G cells expressing V5-tagged wild type or S21T mutant THAP1 proteins confirmed that genetically identified point mutations within the THAP domain abolish THAP1/*TOR1A* interactions *in vivo* (Fig 3C). Interestingly, the THAP1 Q154fsX180 mutant also failed to associate with the *TOR1A* promoter in the ChIP-qPCR assay, despite the fact that this THAP1 mutant contains an intact DNA-binding domain. The THAP1 Q154fsX180 mutant protein is predicted to lack the nuclear localization sequence (NLS) required to enter the nucleus. Accordingly, immunofluorescence studies in both T98G (Fig 3D) and 293T (data not shown) cells indicated that this mutant THAP1 protein localizes to the cytoplasm, contrary to the wild type or S21T point mutant THAP1 proteins that exhibit nuclear localization. We concluded that the different THAP1 mutations described in dystonia type 6 patients, which affect either the DNA-binding domain or remove the NLS, have the same functional consequence i.e. the abrogation of THAP1 binding to *TOR1A in vivo*. This may explain the lack of genotype-phenotype correlations in dystonia type 6 patients to date.

To determine whether THAP1 modulates *TOR1A* expression, we analyzed *TOR1A* mRNA levels in lymphoblast-derived cell lines from dystonia type 6 patients. However, we did not detect a specific modulation of torsinA expression in the lymphoblast cell lines from patients compared to unaffected controls (Supplementary Fig 2). In addition, we did not detect any significant effect on torsinA expression after over-expression of THAP1 by transfection and by retroviral delivery in 293T cells and HUVECs, respectively, despite the fact high levels of THAP1 were measured by western blot analysis and qPCR (Supplementary Fig 3). Similarly, no significant effects on torsinA mRNA levels were found after knockdown of THAP1 in HUVECs (Supplementary Fig 3), suggesting regulation may only occur in specific regions of the brain or during development.

Finally, we analyzed THAP1 mRNA levels in DYT1 patient fibroblasts, but did not detect a change in THAP1 expression in these cells when compared to healthy controls (Supplementary Fig 4).

Discussion

Despite the identification of several gene loci associated with dystonia, its pathophysiology remains poorly understood. *TOR1A* and *THAP1* are the only genes identified thus far for primary dystonia. In this study, we demonstrate that THAP1 protein physically interacts with the *TOR1A* gene promoter both *in vitro* and *in vivo*. Our findings provide the first evidence for what may be a functional link between two forms of primary dystonia and suggest the mechanism of transcriptional dysregulation as a cause of primary dystonia.

While we show in the present study that dystonia type 1 and 6 intersect in a common pathway, the difference in age- and distribution-related spectrum of primary dystonia remains obscure. The fact that THAP1 is an upstream component of the pathway may explain at least in part some differences observed between both phenotypes, including the more frequent oral presentation of patients with dystonia type 6. THAP1 may regulate other potential gene targets, which could also influence phenotype. Nevertheless, even within one particular dystonia type, the age of onset and the distribution of the disease vary greatly, indicating that other factors must be involved in the development of symptoms.

A single transcription factor can act as both a repressor and enhancer of transcription, depending on the genomic context, tissue and/or cell type specificity, and availability of co-factors[16, 17]. Although both THAP1 and torsinA are ubiquitously expressed, they exhibit regional specificity. It is possible, therefore, that disruption of the THAP1 – *TOR1A* interaction may be relevant to disease only in the central nervous system, and perhaps only in specific regions and neuronal subtypes[18-20]. This possibility is supported by our observations that torsinA mRNA levels were not modulated in non-neuronal cells after THAP1 knockdown or overexpression, and in lymphoblastoid cell lines from dystonia type 6 patients. THAP1 regulation of *TOR1A* remains to be tested in brains from dystonia type 6 patients and in mouse models of dystonia type 6, neither of which is currently available. Despite the fact this question remains to be addressed, the present study offers important new insights into molecular mechanisms of primary dystonia pathogenesis and raises the possibility that novel therapeutics targeting this common pathway may be effective for the treatment of different forms of dystonia. Pharmacologic therapy for dystonia remains unsatisfactory[21], and transcription factors are selectively enriched as drug targets among the current FDA approved drugs[22], making THAP1 an obvious and attractive target. Moreover, THAP1 is known to interact with other proteins[23, 24] and characterization of further transcriptional targets, particularly other genes related to dystonia, and interactors may lead to discovery of additional drug targets for dystonia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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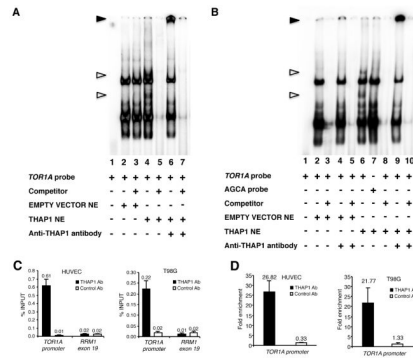
Figure 1.
THAP1 binds the TOR1A promoter in vitro and in vivo. (A,B) Nuclear extracts (NE) from 293T cells transfected with wildtype human THAP1 were subjected to electrophoretic mobility shift assay (EMSA) using the TOR1A promoter region −159/−80 as a probe in A, or the same probe mutated in the THAP1 core consensus site (AGCA probe) in B. Unfilled triangles indicate specific nuclear complexes. Filled triangles indicate the supershifted bands. (C,D) ChIP-qPCR assays were used to analyze association of THAP1 with the TOR1A promoter in vivo. Cross-linked chromatin from HUVEC primary cells or T98G cells was subjected to immunoprecipitation with antibodies against THAP1 (THAP1 Ab1) or negative control antibodies (Control Ab). The human RRM1-exon 19 genomic region was used as a control genomic region. (C) Immunoprecipitated DNA was quantified by qPCR using the percent of input method. (D) Fold enrichment of THAP1 on the TOR1A promoter was calculated by dividing the amount of TOR1A promoter DNA precipitated by anti-THAP1 antibodies to the amount of DNA precipitated from the RRM1-exon 19 genomic region. ChIP results are shown as means with SD from three separate datapoints.
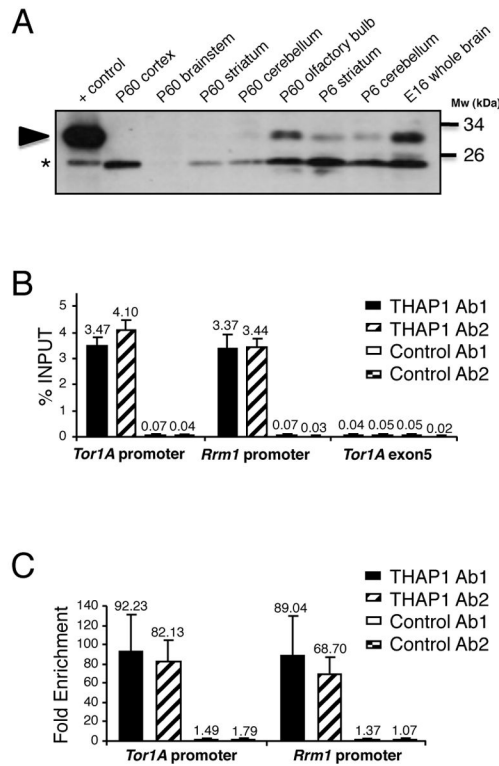
Figure 2.
Endogenous THAP1 is bound to the Tor1A promoter in mouse brain. (A) Immunoblot analysis of THAP1 expression using anti-THAP Ab2 in nuclear fractions (80 μg/lane) of wildtype mouse brains structures obtained at indicated ages. Nuclear extracts (10 μg) of cells transfected with THAP1 were used as positive control. Arrowhead indicates band of the predicted size for THAP1. *Nonspecific immunoreactive band. (B,C) Cross-linked chromatin from adult mouse brain was subjected to immunoprecipitation with two distinct THAP1 (THAP1 Ab1 and THAP1 Ab2) and negative control antibodies (Control Ab1 and Control Ab2). The mouse Rrm1 promoter and Tor1A-exon 5 genomic region were used as positive and negative control regions, respectively. Immunoprecipitated DNA was quantified by qPCR (B) and fold enrichment (C) was calculated by dividing the amount of Tor1A or Rrm1 promoter DNA precipitated by anti-THAP1 antibodies to the amount of DNA precipitated from the Tor1A-exon 5 genomic region. Results are shown as means with SD from three separate datapoints.
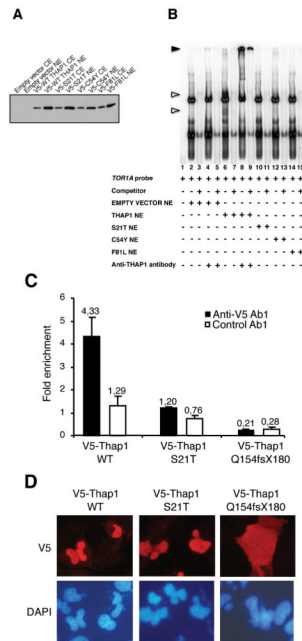
Figure 3.
Causative mutations of DYT6 dystonia disrupt the THAP1/TOR1A interaction. (A)
Immunoblot with monoclonal anti-V5 antibody showing similar levels of expression of the
wildtype and mutant THAP1 proteins in cytoplasmic and nuclear extracts of 293T cells
transfected with the indicated expression vectors. (B) Electrophoretic mobility shift assay
(EMSA) showing that DYT6 patient point mutations disrupt physical interaction between
THAP1 and TOR1A promoter region. Nuclear extracts from 293T cells transfected with the
indicated expression vectors were used. Unfilled triangles indicate specific nuclear
complexes. Filled triangles indicate the supershifted bands. (C,D) Cross-linked chromatin
from T98G cells transfected with the indicated expression vectors was subjected to
immunoprecipitation with anti-V5 or control antibodies. Immunoprecipitated DNA was
quantified as described above (see Fig 1 legend) and fold enrichment of wildtype THAP1 or
mutant proteins over the TOR1A promoter (C) was calculated by dividing the amount of
TOR1A promoter DNA precipitated by anti-V5 antibodies to the amount of DNA
precipitated from the RRM1-exon 19 genomic region. The results are shown as means ± SD
from three separate datapoints. (D) Transfected T98G cells were analyzed by indirect
immunofluorescence microscopy with anti-V5 antibodies (red). DNA was counterstained
with DAPI (blue)