The Neurodegenerative Disease Protein Ataxin-1 Antagonizes the Neuronal Survival Function of Myocyte Enhancer Factor-2*\textsuperscript{S1,5}

Timothy A. Bolger\textsuperscript{1,1}, Xuan Zhao\textsuperscript{1,1}, Todd J. Cohen\textsuperscript{1}, Chih-Cheng Tsai\textsuperscript{1}, and Tso-Pang Yao\textsuperscript{1,2}

From the \textsuperscript{1}Department of Pharmacology and Cancer Biology, Duke University, Durham, North Carolina 27710 and the \textsuperscript{2}Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Ataxin-1 is a neurodegenerative disorder protein whose mutant form causes spinocerebellar ataxia type-1 (SCA1). Evidence suggests that ataxin-1 may function as a transcription repressor. However, neither the importance of this putative transcriptional repression activity in neural cytotoxicity nor the transcriptional targets of ataxin-1 are known. Here we identify the MEF2-HDAC4 transcriptional complex involved in neuron survival as a target of ataxin-1. We show that ataxin-1 binds specifically to histone deacetylase-4 (HDAC4) and MEF2 and colocalizes with them in nuclear inclusion bodies. Significantly, these interactions are greatly reduced by the S776A mutation, which largely abrogates the cytotoxicity of ataxin-1. Supporting the importance of these interactions, we show that wild type ataxin-1 represses MEF2-dependent transcription, whereas the S776A mutant is less potent. Furthermore, overexpression of MEF2 can partially reverse cytotoxicity caused by ataxin-1. Our results identify the MEF2-HDAC4 complex as a target for ataxin-1 transcriptional repression activity and suggest a novel pathogenic mechanism whereby ataxin-1 sequesters and inhibits the neuronal survival factor MEF2.

Spinocerebellar ataxia-1 (SCA1)\textsuperscript{3} is an autosomal-dominant neurodegenerative disease that leads to the loss of Purkinje neurons in the cerebellum as well as other neuron subsets (1). SCA1 is one of at least nine polyglutamine diseases caused by mutant proteins with an expanded polyglutamine (polyQ) tract (2). These mutant proteins are prone to the formation of toxic aggregates that are likely to be a major cause of neuronal cell death (reviewed in Ref. 3). In SCA1, ataxin-1 has been identified as the polyglutamine disease protein (4). Although its physiological function is not known with certainty, ataxin-1 interacts with the transcription corepressor silencing mediator of retinoid and thyroid hormone receptor and histone deacetylase HDAC3, and it can repress transcription when tethered to DNA (5). Interestingly, these interactions are mediated by a conserved domain termed the AXH, which has also been reported to contribute to aggregation of ataxin-1 (6, 7). Ataxin-1 also interacts with another AXH-containing protein named Boat (Bother of ataxin-1), which may be a transcriptional repressor as well (6). These findings, as well as a recent report of ataxin-1 in complexes with the repressor Capicua (CIC), indicate an activity of ataxin-1 in transcriptional repression (8). However, to what degree this transcriptional repression activity is important for ataxin-1-dependent pathogenesis and what specific transcriptional pathways are targeted by ataxin-1 remain unknown. Answers to these questions may add to our understanding of how ataxin-1 induces neuron death and reveal targets that might be critical for SCA1 pathogenesis.

Disease-associated ataxin-1 variants have an expansion of the polyglutamine tract, and they form nuclear inclusion bodies in cultured cells and in neurons from affected patients and transgenic mice (1). However, other characteristics besides the length of the polyglutamine tract strongly affect disease development. Overexpression of a short polyQ form of ataxin-1 in fly and mouse models also leads to the formation of nuclear inclusions and a neurodegenerative phenotype, albeit a milder one (1, 9). Furthermore, elegant studies have revealed that phosphorylation at serine 776 is a critical modulator for ataxin-1 toxicity (10, 11). The phosphorylation of serine 776 was shown to recruit the phospho-binding protein 14-3-3, resulting in increased ataxin-1 stabilization, aggregation, and toxicity (11). Remarkably, mutation of this serine to alanine (S776A) severely impairs the ability of ataxin-1 to elicit a prominent neurodegeneration phenotype (10). Although the serine 776 phosphorylation status robustly affects the potency of ataxin-1 to induce cytotoxicity and neurodegeneration, the mechanism by which this phosphorylation affects pathogenesis is not well understood.

Histone deacetylase-4 (HDAC4), a member of the class Ila histone deacetylases, has previously been identified as a regulator of neuronal cell death (12). HDAC4 actively shuttles between the nucleus and cytoplasm in response to specific signaling events (13). Under conditions of activity-dependent neuron death, HDAC4 translocates to the nucleus, and there it promotes cell death through its transcription repression activity (12). This is accomplished at least in part by binding and...
inhibiting the transcription factor MEF2. MEF2 acts as a neuronal survival factor, and its inhibition has been linked to several different kinds of neuron death (14–16). Although these studies indicate that HDAC4 and MEF2 are regulators of activity-dependent cell death, whether this transcriptional complex is involved in other types of neuron death, such as those associated with neurodegeneration, is not known.

In this study, we describe a functional interaction between ataxin-1, HDAC4, and MEF2. We show that ataxin-1 binds HDAC4 and MEF2, and they colocalize to ataxin-1-induced nuclear inclusion bodies. Functionally, we present data that ataxin-1 acts as a repressor of MEF2-dependent transcription. Importantly, the less toxic ataxin-1 S776A mutant shows a markedly reduced interaction with HDAC4 and MEF2 and compromised transcriptional repressive activity. Lastly, we show that overexpression of MEF2 is able to partially rescue cellular toxicity caused by ataxin-1. Our study identifies the MEF2-HDAC4 transcriptional complex as a target of ataxin-1, and we suggest that repression of MEF2 activity may contribute to ataxin-1-induced neurotoxicity and neurodegeneration.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The HDAC4 polyclonal antibody, ab186, was described previously (13). Monoclonal antibodies against GFP, FLAG, and MEF2 were from Roche Applied Science, Sigma-Aldrich, and BD Biosciences, respectively. Secondary antibodies were from Jackson ImmunoResearch. pCDNA-HDAC4 and pCDNA-MEF2D plasmids were as described in Ref. 17.

Cell Lines and Transfections—Cerebellar granule neurons were obtained from P7-P9 mice as described (12). Cerebellar granule neuron (CGN) medium consisted of Neurobasal with 5% fetal bovine serum, B27 supplement, sodium pyruvate, L-glutamine, penicillin/streptomycin, 0.6 mg/ml glucose, and 25 mM KCl. After 1 day in culture, 10 μM cytosine arabinoside was added to cells to inhibit non-neuronal cells. CGNs were transfected immediately following isolation using the mouse neuron Nucleofector kit (Amaxa), and cells were then cultured for 2 days before harvesting. CAD cells, a variant of Cath.a cells, were cultured in 8% fetal bovine serum in DMEM-F12. Differentiation medium consisted of DMEM-F12 without serum. Cos-7 and 293T cells were cultured in 10% fetal bovine serum in DMEM. C2C12 cells were cultured in 20% fetal bovine serum in DMEM. Transfections were performed with FuGENE 6 (Roche Applied Science) per the manufacturer’s instructions.

Immunofluorescent Staining—Samples were stained for immunofluorescence essentially as described (13). When samples were harvested, cover slips were rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde. They were washed and then permeabilized with 0.2% Triton X-100. Samples were blocked with 5% normal goat serum and incubated with primary antibody overnight at 4 °C. Sections were then washed, incubated with secondary antibody, washed again, stained with Hoechst dye, and mounted with Fluoromount-G. CGN images in Fig. 1A were captured with a Zeiss LSM410 confocal microscope with ×63 objective lens. All other images were taken with a Zeiss Axioimager microscope with ×63 objective.

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Immunoprecipitations and Immunoblotting—For immunoprecipitations, cells were lysed in either modified radioimmune precipitation buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitors) for HDAC4 immunoprecipitations or NETN (170 mM NaCl, 5 mM EDTA, 50 mM Tris, 1% Nonidet P-40, and protease inhibitors) for MEF2 immunoprecipitations. 0.8–1.2 mg of total protein were rocked 5 h to overnight at 4 °C with primary antibody, rabbit α-mouse IgG, and protein A-conjugated beads. Beads were spun down and then washed three times with lysis buffer, and then 2X sample buffer was added. Samples were then boiled for 10 min, and beads were removed by centrifuging. Samples were then run on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted. For band intensity quantitation, NIH ImageJ software was used to analyze the bands. The values for the coimmunoprecipitated ataxin-1 were normalized to the ataxin-1 protein levels in the respective whole cell lysates. The values for ataxin1-82Q alone (see Fig. 2B) and for ataxin1-2Q (see Fig. 3D) were set to 100.

Luciferase Assays—Luciferase assays were performed in triplicate essentially as described (13). Cos7 cells were transfected with a luciferase reporter containing three tandem binding sites for MEF2 and expression plasmids for MEF2D and ataxin-1. The assay results were normalized by total protein concentration as determined by Bradford assay. Relative activity is compared with reporter alone. The means from three independent experiments are shown, and in Fig. 4, error bars represent S.E.

Cytotoxicity Assay—For the cytotoxicity assay, CAD cells in 24-well plates were transfected with GFP, ataxin-1, and MEF2. After 24 h, the cells were placed in differentiation medium for an additional 24 h. Then the samples were subjected to the Cytotox 96 non-radioactive cytotoxicity assay (Promega), which compares lactate dehydrogenase levels in the medium from ruptured, dying cells with the levels in lysates from the remaining healthy cells. The medium from each sample was removed, centrifuged briefly, and then diluted 1:3 for the assay. Cells remaining on the plates were lysed in buffer containing 0.9% Triton for 45 min at 37 °C. Lysates were transferred to tubes, centrifuged briefly, and diluted 1:10 for the assay. 50 μl of diluted medium or lysate was mixed in a 96-well plate with an equal amount of substrate mix and incubated for 20–30 min until color change was evident. Absorbance was then read at 490 nm on a plate reader. Relative values were determined by subtracting background and multiplying by the dilution factor, and the percentage of cell death was determined by dividing the value derived from the medium by the lysate value for each sample. Samples were performed in duplicate or triplicate, and data were collected from four independent experiments. The mean for each is shown, and in Fig. 4, error bars represent standard error of the mean.

RESULTS

As we have shown previously, HDAC4 translocates to the nucleus under conditions of neuron death (12). We wished to examine whether this translocation also occurred in pathological conditions of neuronal cell death, such as death caused by expanded polyglutamine-tract proteins in neurodegenerative
To this end, we transfected CGNs with a disease-associated form of ataxin-1 that contains a glutamine tract of 82 residues (ataxin-1-82Q) and examined the localization of HDAC4 with a specific antibody (13). As shown in Fig. 1A, in a control GFP transfection, HDAC4 was primarily localized to the cytoplasm. In contrast, transfected ataxin-1-82Q caused HDAC4 nuclear accumulation (Fig. 1A). Unexpectedly, in those cells, a portion of the nuclear HDAC4 was also concentrated to ataxin-1 inclusion bodies (see inset).

To confirm the recruitment of HDAC4 to nuclear inclusion bodies, we transfected CAD cells, a catecholaminergic cell line derived from mouse neuroblastoma, with GFP-ataxin-1-82Q and stained them with HDAC4 and GFP antibodies (18). As in CGNs, endogenous HDAC4 staining in these cells also colocalized with ataxin-1-82Q inclusions (Fig. 1B). HDAC4 can also be found at nuclear inclusion bodies formed by a 2Q form of ataxin-1, indicating that the recruitment of HDAC4 is not strictly dependent on polyglutamine length (data not shown). Similar colocalization can be observed with ectopically expressed FLAG-tagged HDAC4 and GFP-ataxin-1, as well as FLAG-ataxin-1-82Q and endogenous HDAC4 (supplemental Fig. 1). Importantly, related family members HDAC1 and HDAC10 were not concentrated to the ataxin-1 inclusion bodies (Fig. 1C and data not shown). These observations show that HDAC4 colocalizes with ataxin-1 inclusion bodies, indicating a specific association of HDAC4 and ataxin-1.

Inclusion body formation and the neural toxicity of ataxin-1 have been shown to be regulated by phosphorylation of serine 776 (10, 11). Mutation of this site to alanine (S776A) results in an ataxin-1 that forms fewer inclusions and has severely reduced neurodegeneration when compared with non-mutated forms. If HDAC4 association with ataxin-1 is related to the disease process, we reasoned that its interaction might be dependent on Ser-776 phosphorylation. To address this possibility, we transfected GFP-ataxin-1-82Q and the 82Q-S776A mutant into CAD cells and stained the cells for GFP and HDAC4 (Fig. 2A). As reported previously, the ataxin-1-S776A mutant can still occasionally form inclusion bodies (11). Intriguingly, HDAC4 is absent from most of these S776A-ataxin-1 inclusion bodies (Fig. 2A, bottom panels), indicating that the association of HDAC4 and ataxin-1 is negatively affected by the S776A mutation. Consistent with the immunolocalization study, a coimmunoprecipitation assay revealed that both ataxin1-2Q and 82Q-ataxin-1 interact with HDAC4 (Fig. 2B). In contrast, the ataxin-1-S776A mutant associated with HDAC4 weakly and below background level. Taken together, these data argue strongly that HDAC4 associates with ataxin-1 and that this binding is modulated by serine 776 of ataxin-1.

To begin to elucidate the functional significance of the association of HDAC4 and ataxin-1, we investigated the potential involvement of MEF2 for two reasons. First, HDAC4 binds MEF2 with high affinity and represses its transcriptional activity (19). Second, MEF2 is well established as a pro-survival factor in neurons (14). As shown in Fig. 3A, endogenous MEF2 was prominently present at ataxin-1 inclusion bodies in CAD cells. This colocalization is specific, as neither estrogen receptor-α nor SP-1 was found in the inclusion bodies (Fig. 3B and data not shown). Importantly, like HDAC4, MEF2 is not recruited to inclusion bodies induced by the ataxin-1-S776A mutation.
mutant (Fig. 3C). Further validating the interaction of ataxin-1 and MEF2, coimmunoprecipitation experiments showed that MEF2 associates with both the 2Q and the 82Q forms of ataxin-1 but much less efficiently (30%) with the ataxin-1-S776A mutant (Fig. 3D). Thus this data demonstrate that MEF2 also associates with ataxin-1 and that ataxin-1-S776A, a mutation associated with less toxicity, has reduced association with both HDAC4 and MEF2.

Because ataxin-1 has the ability to act as a transcriptional repressor, we hypothesized that ataxin-1 may interact with HDAC4 and MEF2 and repress MEF2-dependent transcription. To address this hypothesis, we transfected Cos-7 cells with a luciferase reporter containing three tandem repeats of MEF2 binding sites and performed luciferase assays. Cotransfection of MEF2 resulted in substantial activation of the reporter (Fig. 4A). The addition of either ataxin-1-2Q or ataxin-1-82Q repressed MEF2-dependent reporter activity, indicating that ataxin-1 can indeed inhibit MEF2. Importantly, the S776A mutant of ataxin-1 was significantly impaired in its ability to repress MEF2 transcription activity. These data indicate that ataxin-1 can act as a transcriptional corepressor of MEF2. Further, the difference in activity between ataxin-1-82Q and the S776A mutant suggests a correlation between the abilities of ataxin-1 to induce cellular toxicity and repress MEF2 activity.

MEF2 is known to be an important factor in neuronal survival (14). Therefore, inhibition of MEF2 by ataxin-1 through its transcription repression activity and its sequestration of MEF2 to inclusions may be important for ataxin-1-induced cell death. To test this hypothesis, we asked whether overexpression of MEF2 can alleviate ataxin-1-induced cell death. To that end, we cotransfected CAD cells with MEF2 and GFP, ataxin-1-82Q, or ataxin-1-82Q-S776A and then differentiated the cells by serum starvation for 24 h. We then performed a colorimetric cytotoxicity assay that measures lactate dehydrogenase activity to determine the percentage of cell death. As shown in Fig. 4B, ataxin-1-82Q (21%) induced about a 3-fold increase in the amount of cell death over GFP controls (<8%), whereas the toxicity of the S776A mutant was considerably lower (11%). Importantly, although cotransfection of MEF2 had little effect on cell death in GFP and S776A mutant samples, it significantly reduced the toxicity of the ataxin-1-82Q by about one-third (15%), indicating that MEF2 overexpression can partially rescue cell death caused by ataxin-1. Transfection of ataxin-1-2Q also induced cell death, although less than the 82Q form, and was largely rescued by MEF2 overexpression (supplemental Fig. 2). These results are consistent with the hypothesis that inhibition of MEF2 contributes to ataxin-1-mediated toxicity.

**DISCUSSION**

Ataxin-1 has previously been shown to bind HDAC3 and the corepressor silencing mediator of retinoid and thyroid hormone receptor, and this finding led to the proposal that ataxin-1 might normally function as a transcriptional corepressor (5). This is also supported by a recent report that ataxin-1 associates with another corepressor, Capicua or CIC (8). Our finding that ataxin-1 interacts with HDAC4 further strengthens this proposition. Importantly, our study identifies the neuronal survival factor MEF2 as the first potential transcriptional target for ataxin-1. This finding provides direct evidence that ataxin-1 indeed has the capacity to inhibit gene expression associated...
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with neuronal survival. It also offers an alternative mechanism by which mutant ataxin-1 can cause cell death through repression of the pro-survival activity of MEF2. Supporting this idea, we show that overexpression of MEF2 can alleviate the cytotoxicity induced by mutant ataxin-1. Together with the known functions of the MEF2/HDAC4 transcriptional complex in neuronal cell death, our study suggests that MEF2 inhibition by ataxin-1 might be important for the pathogenesis of SCA1.

We report that HDAC4 and MEF2 associate with both long and short forms of ataxin-1, indicating that their association is not strictly dependent on polyQ length. Although an expanded polyQ tract is the hallmark of polyglutamine diseases and no doubt important, it has become increasingly clear that the pathogenesis of these diseases involves more than just the expansion of the polyglutamine tract itself (20). In the case of ataxin-1, overexpression of the protein with even a short polyQ can cause a neurodegenerative phenotype in animal models (1, 9). Our findings provide further support for an emerging concept that the normal functions and interactions of the polyQ disease proteins are crucial to their pathology (21). Consistent with this idea, during revision of this work, Lam et al. (8) reported that both the short and the long-Q forms of ataxin-1 associate with CIC transcriptional corepressor complexes, and this interaction modulates the degenerative effects of ataxin-1 in Drosophila. In this light, it is reasonable to suggest that ataxin-1 can associate with HDAC4 and MEF2 as part of its normal function but that during SCA1 pathology, overabundance of ataxin-1 or other aberrant regulation of the disease protein causes inappropriate inhibition of MEF2, contributing to cytotoxicity.

A further illustration of the importance of regions outside the polyQ domain is Ser-776 phosphorylation. The S776A mutant of ataxin-1 is much less prone to aggregate and is inefficient at inducing neurodegeneration in a transgenic model (10). Remarkably, we found that the ability of ataxin-1 to interact with HDAC4 and MEF2 and inhibit MEF2 activity is severely compromised by the S776A mutation, providing a potential novel mechanism explaining the differential toxicity modulated by Ser-776 phosphorylation. Thus, the neurotoxic potency of ataxin-1 is correlated with its ability to interact with the MEF2-HDAC4 transcriptional complex. Interestingly, the S776A mutation also altered association of ataxin-1 with the CIC-containing complexes (8). It would be of interest to determine in future studies whether the interactions of CIC and HDAC4/MEF2 with ataxin-1 are functionally related.

Inhibition of MEF2 by ataxin-1 may occur by multiple, non-exclusive mechanisms. The observation that MEF2 is recruited into ataxin-1-dependent inclusions suggests that part of
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Under this scenario, high levels of ataxin-1 or other abnormal regulation of ataxin-1 could cause constitutive, aberrant inhibition of MEF2 transcriptional activity, down-regulating gene expression important for neuronal survival. Accordingly, restoring MEF2 transcriptional activity, for example by HDAC inhibitors, could be beneficial for SCA1 patients.

Although HDAC4 interacts with ataxin-1, the specific role for HDAC4 in SCA1 pathogenesis remains unclear. Given that HDAC4 and ataxin-1 have both been reported to interact with 14-3-3, we speculated that 14-3-3 might be important for the interaction (11, 13, 27). However, a 14-3-3 binding mutant of HDAC4 is still recruited to ataxin-1 inclusions, and overexpression of 14-3-3 does not appear to alter the HDAC4/ataxin-1 interaction (supplemental Fig. 3). Another possibility is that HDAC4 might recruit MEF2 to ataxin-1; however, we found that MEF2 is still able to localize to ataxin-1 inclusion bodies and coimmunoprecipitate ataxin-1 in HDAC4 knockdown cells (data not shown). This result could be due to residual HDAC4 or compensation by other closely related class IIa HDACs. We note that we were unable to show HDAC4 localization to inclusion bodies from ataxin-1 transgenic mice (not shown). However, HDAC4 has been reported to be present in the inclusion bodies found in neuronal intranuclear inclusion disease (28).

Regardless, the connection between ataxin-1 and HDAC4 is intriguing, as HDAC4 is required for efficient cerebellar granule neuron death induced by depolarization or excitotoxic conditions (12). It is tempting to speculate that HDAC4 might be involved in neuronal cell death induced by ataxin-1. Such a conclusion would be of major significance and have potential clinical implications for SCA1 pathogenesis. Indeed, histone deacetylase inhibitors have been used to treat neurodegenerative disease in a number of animal models (29–31). It would be of great interest to determine whether HDAC4 is the molecular target of these inhibitors.

The data in this study show that ataxin-1 interacts with HDAC4 and MEF2. We suggest that ataxin-1 and HDAC4 are physiological binding partners and can repress MEF2 in normal conditions when needed. However, the dysregulation of ataxin-1 that occurs in the disease process likely causes it to acquire either additional aberrant functions or inappropriate manifestations of normal functions. We propose that part of ataxin-1 pathogenesis may involve the sequestration of MEF2 into nuclear inclusions and/or inhibition of MEF2 through transcriptional repression, in either case preventing MEF2 from promoting a neuronal survival transcription program, thereby contributing to eventual neuron cell death.

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