Atrophin-1, the Dentato-rubral and Pallido-luysian Atrophy Gene Product, Interacts with ETO/MTG8 in the Nuclear Matrix and Represses Transcription

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Abstract. Dentato-rubral and pallido-luysian atrophy (DRPLA) is one of the family of neurodegenerative diseases caused by expansion of a polyglutamine tract. The drpla gene product, atrophin-1, is widely expressed, has no known function or activity, and is found in both the nuclear and cytoplasmic compartments of neurons. Truncated fragments of atrophin-1 accumulate in neuronal nuclei in a transgenic mouse model of DRPLA, and may underlie the disease phenotype.

Using the yeast two-hybrid system, we identified ETO/MTG8, a component of nuclear receptor corepressor complexes, as an atrophin-1-interacting protein. When cotransfected into Neuro-2a cells, atrophin-1 and ETO/MTG8 colocalize in discrete nuclear structures that contain endogenous mSin3A and histone deacetylases. These structures are sodium dodecyl sulfate–soluble and associated with the nuclear matrix. Cotransfection of ETO/MTG8 with atrophin-1 recruits atrophin-1 to the nuclear matrix, while atrophin-1 and ETO/MTG8 cofractionate in nuclear matrix preparations from brains of DRPLA transgenic mice. Furthermore, in a cell transfection–based assay, atrophin-1 represses transcription. Together, these results suggest that atrophin-1 associates with nuclear receptor corepressor complexes and is involved in transcriptional regulation.

Emerging links between disease-associated polyglutamine proteins, nuclear receptors, translocation–leukemia proteins, and the nuclear matrix may have important repercussions for the pathobiology of this family of neurodegenerative disorders.

Key words: trinucleotide repeats • neurodegenerative diseases • cerebellar nuclei • nuclear matrix • myeloid leukemia

Introduction

Dentato-rubral and pallido-luysian atrophy (DRPLA) is one of at least eight neurodegenerative disorders caused by the expansion of a polyglutamine tract within a disease-associated protein (Ross, 1995; Gusella and MacDonald, 1996; Paulson and Fischbeck, 1996). There is a critical threshold for disease at ~40 glutamines; expansion of the polyglutamine tract beyond this threshold gives a neurotoxic protein. The mechanisms underlying neurotoxicity are not fully understood. The DRPLA gene product, atrophin-1, has no known function or biological activity, and is found in both the nuclear and cytoplasmic compartments of neurons (Yazawa et al., 1995; Schilling et al., 1999). Truncated fragments of atrophin-1, containing the expanded polyglutamine tract, have been shown to accumulate in populations of neuronal nuclei, both in a transgenic mouse model of DRPLA and in human postmortem DRPLA patient brain tissue (Schilling et al., 1999). These fragments may underlie the pathogenesis of DRPLA.

There is evidence to suggest that the nucleus may be the...
primary site of cellular dysfunction in other members of this family of diseases (Klement et al., 1998; Saudou et al., 1998). Polyglutamine-containing proteins have an inherent ability to aggregate when the length of the polyglutamine tract exceeds the aforementioned critical threshold (Scherzinger et al., 1997). The threshold for aggregation closely correlates with the threshold for disease, and neuronal intranuclear inclusion bodies have been identified as a pathological hallmark in these disorders (Ross, 1997). Nuclear inclusions are macromolecular structures, some of which are ubiquitinated, and contain molecular chaperones and components of the proteasome in addition to the expanded polyglutamine protein (Cummings et al., 1998; Chai et al., 1999). Other nuclear proteins that contain glutamine repeats, such as TATA-binding protein and CREB-binding protein (CBP; Kazantsev et al., 1999) can be recruited into these structures (Perez et al., 1998). It is not clear whether the inclusions are directly neurotoxic. In the cases of huntingtin and ataxin-1, it has been suggested that nuclear localization of the mutant protein is more important than the presence of macromolecular aggregates in determining neurotoxicity (Klement et al., 1998; Saudou et al., 1998). Whether aggregation is critical or not, the nucleus is strongly implicated as a major site of neuronal dysfunction.

As truncated fragments of atrophin-1 accumulate in neuronal nuclei in a transgenic mouse model of DRPLA, we have sought to identify nuclear processes in which atrophin-1 is involved. Using the yeast two-hybrid system, we now demonstrate that atrophin-1 interacts with a protein known as ETO/MTG8. ETO/MTG8 is a member of a family of highly conserved proteins, and was first identified as the fusion partner with AML1 in t(8;21) acute myeloid leukemia (Erickson et al., 1992; Nisson et al., 1992; Miyoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of highly conserved proteins, and was first identified as the fusion partner with AML1 in t(8;21) acute myeloid leukemia (Erickson et al., 1992; Nisson et al., 1992; Miyoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993). The ETO/MTG8 moiety of the chimeric AML1-ETO/MTG8 fusion diverts the AML1 moiety from AML1 subnuclear domains (McNeil et al., 1999) and recruits the nuclear receptor corepressor (N-CoR)/mSin3 histone deacetylase (HDAC) complex (Gelmetti et al., 1998; Yoshi et al., 1993).

**Materials and Methods**

**Yeast Two-hybrid Screening**

Nucleotides 76-1438 of atrophin-1 (encoding lysine 2 to asparagine 455) were amplified by PCR using EXPA ND high fidelity enzyme mix (Roche) using the following primers: 5’- ACGTGCACCGGAGAAGA CAGCAGCA - and 5’-GGATCCTAGCTG GCCGCTTTA ATGCTGTTGGCTA -3’, and pCDNA 3A-T-F26L as template. The PCR product was digested with SalI and NotI and ligated into the GAL4 DNA-binding domain (DBD) vector pPC97 (Chevray and Nathans, 1992). DNA sequencing confirmed in-frame fusion of atrophin-1 to the GAL4 DBD. The resulting vector, pPC97-A1-2-455, was cotransformed into yeast strain Y130 with the GAL4 activation domain vector pPC86 (Chevray and Nathans, 1992) to confirm that it did not activate the His and β-galactosidase reporter genes in the absence of an interaction partner. pPC97-A1-2-455 was used to screen a human cerebellum cDNA library in pPC86 as described elsewhere (Wood et al., 1998). The library plasmids were isolated from His’β-gal’ colonies, retransformed into Y130 with pPC97-A1-2-455 to confirm isolation of putative interactors, and sequenced.

**Immunopurification of Atrophin-1**

A nitrobody A PG 840 was raised against synthetic peptide DRPLA-425 corresponding to residues 425-439 of human atrophin-1 at Cocalico Biologicals Inc., Reamstown, PA. This peptide is 100% conserved between human and mouse and was previously used to produce antibody A142 in rabbit (Wood et al., 1998). A nitrobody were purified from serum on a Sulfolink-DRLA-425 column and coupled to CarboLink gel (Pierce Chemical Co.). Brain extracts were prepared by homogenizing mouse brains in 10 vol of PBS supplemented with 0.5% Igepal CA-630, 1 mM PM Sf, and complete protease inhibitor cocktail (Roche) followed by centrifugation at 10,000 g for 10 min at 4°C. A trophin-1 was immunopurified from brain extracts with 100 μg of A PG 840 coupled to 400 μl of CarboLink gel. Control immunopurifications were performed with 200 μg of goat IgG coupled to 400 μl of CarboLink gel. Purified complexes were washed three times with homogenization buffer and analyzed by immunoblotting with anti-atrophin-1 antibody A142 and anti-ETO/MTG8 antibody 2174 (Sacchi et al., 1998).

**Cell Transfection and Immunofluorescence Microscopy**

Neuro-2a cells were cultured in 1:1 DMEM/OPTI-MEM 1 supplemented with 5% FBS and antibiotics. For immunofluorescence microscopy, cells were transiently transfected in 4-well chamber slides with various combinations of pCDNA 3, pCDNA 3A-T-F26L, pCDNA 3A-T-F65Q, and pCDNA 3-FLAG-ETO/MTG8 (total 0.2 μg DNA well) using Lipofectamine PLUS (Life Technologies). 24 h after transfection, they were fixed in 31% methanol/acetone for 10 min at −20°C. Immunostaining was performed using rabbit polyclonal anti-atrophin-1 antibody AP 342 (Wood et al., 1998), goat polyclonal anti-atrophin-1 antibody A PG 840, mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich), goat polyclonal antithistone deacetylase 1 (HDAC1) antibody C-19, goat polyclonal histone deacetylase 2 (HDAC2) antibody C-19, rabbit polyclonal anti-Sin3A antibody AK-11, goat polyclonal anti-N-CoR antibody C-20, mouse monoclonal anti-promyelocytic leukemia protein (PLM) antibody PMG-3 (all from Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-PML antibody (a kind gift from Robert Redner, University of Pittsburgh, Pittsburgh, PA), and rabbit polyclonal antiubiquitin antibody (Dako). FITC- and Cy3-labeled secondary antibodies (all from donkey) were from Jackson Immunol Research Laboratories. Immunofluorescence was observed using a Zeiss LSM 410 confocal imaging microscope.

**Immunoblotting**

Neuro-2a cells were cultured in 10-cm-diameter culture dishes and transfected with various combinations of pCDNA 3, pCDNA 3-A-T-F26L, pCDNA 3-A-T-F65Q, and pCDNA 3-FLAG-ETO/MTG8 (total 1 μg DNA well) using Lipofectamine PLUS (Life Technologies). 24 h after transfection, they were washed once with PBS and scraped into 0.5 ml of 20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 1 mM PM Sf, supplemented with complete protease inhibitor cocktail (Roche). A new equal volume of 2× Laemmli sample buffer was then added, and the samples were heated at 70°C for 5 min, sonicated for 20 s, and microfuged for 1 min before loading. Proteins were resolved in a 4-15% polyacrylamide gel and immunoblotted for atrophin-1 with AP142 and FLAG-ETO/MTG8 with monoclonal antibody M2.
pellet was collected by low-speed centrifugation and extracted sequentially with (i) 10 mM Pipes (pH 6.8), 250 mM ammonium sulfate, 300 mM sucrose, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PMSF, 2 mM VRC, and 1× EDTA-free complete protease inhibitor cocktail; (ii) 10 mM Pipes (pH 6.8), 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% Triton X-100, 200 U/ml DNase I, 1 mM PMSF, 2 mM VRC, and 1× EDTA-free complete protease inhibitor cocktail; and (iii) 10 mM Pipes (pH 6.8), 300 mM sucrose, 2 M NaCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PMSF, 2 mM VRC, and 1× EDTA-free complete protease inhibitor cocktail. All extractions were for 5 min at 4°C except for Dnase I digestion, which was performed at 32°C for 30 min. Nuclear matrices were resuspended in 1 ml of SDS-PAGE sample buffer, heated at 70°C for 5 min, and then sonicated for 20 s. Volumetric equivalents of the supernatants from each extraction were immunoblotted for atrophin-1 and FLAG-ETO/MTG8 with antibodies A142 and M2.

For nuclear matrix preparations from brain, nuclei were isolated from DRPLA transgenic mice as described elsewhere (Schilling et al., 1999), and the nuclear matrix was prepared from the isolated nuclei following the same protocol as for transfected cells, except that the final wash with 2 M NaCl was omitted. The subnuclear fractions were immunoblotted for atrophin-1 and ETO/MTG8 with antibodies A142 and M2.

Transcriptional Assays

Full-length atrophin-1 cDNA s, encoding 26 and 65 consecutive glutamines, respectively, were amplified by PCR using EXPAND high fidelity enzyme mix (Roche) with the following primers: 5'-GCTCTAGAAGAATATTGAGACACGCAAGAAATGAGACACGCAAGAAT-3' and 5'-GGGGGCTAGAGGAGGGGTCCTCA-GGTCCTCAAT-3', and pcDNA3-A1-at-FL-26Q and pcDNA3 A1-at-FL-65Q as templates. The products were digested with XbaI and KpnI and ligated as templates. The products were digested with XbaI and KpnI and ligated into the eukaryotic GAL4 DBD fusion protein expression vector pFA-CMV. DNA sequencing confirmed in-frame fusion of atrophin-1 to the GAL4 DNA-binding construct. 1 μg of GAL4 DNA-binding construct with empty pFA-CMV was cotransfected into Neuro-2a cells cultured in Neurobasal medium with 10% FBS, 300 μM sucrose, 0.5% Triton X-100, 200 U/ml DNase I, 1 mM PMSF, 2 mM VRC, and 1× EDTA-free complete protease inhibitor cocktail; and (iii) 10 mM Pipes (pH 6.8), 300 mM sucrose, 2 M NaCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PMSF, 2 mM VRC, and 1× EDTA-free complete protease inhibitor cocktail. All extractions were for 5 min at 4°C except for Dnase I digestion, which was performed at 32°C for 30 min. Nuclear matrices were resuspended in 1 ml of SDS-PAGE sample buffer, heated at 70°C for 5 min, and then sonicated for 20 s. Volumetric equivalents of the supernatants from each extraction were immunoblotted for atrophin-1 and FLAG-ETO/MTG8 with antibodies A142 and M2.

For immunocytochemistry of isolated nuclear matrix preparations, Neuro-2a cells were grown in 2-well chamber slides and transfected with atrophin-1 and FLAG-ETO/MTG8 cDNAs using Lipofectamine PLUS. 24 h after transfection, cells were rinsed with PBS and subjected to the series of extractions described above. After the final extraction, nuclear matrices were immunoblotted for atrophin-1 and ETO/MTG8 with antibodies AP142 (Wood et al., 1998) and M2 monoclonal antibody.

Results

Identification of ETO/MTG8 as an Atrophin-1–interacting Protein

3 × 10<sup>5</sup> clones of a human cerebellum cDNA library in pPC86 were screened with pPC97 containing various insert clones containing cDNA encoding residues 1–598 of the Huntington's disease gene product, huntingtin (with both 18 and 81 glutamines); and various pPC97 atrophin-1 constructs (encoding amino acids 2–455, 413–731 with 26Q, 413–731 with 65Q, 640–1184, 2–1184 with 26Q, and 2–1184 with 65Q respectively). Only yeast cotransformations with ETO/MTG8 and the NH2 terminus of atrophin-1 or full-length atrophin-1 constructs were His<sup>+</sup>/β-gal+ , demonstrating that ETO/MTG8 interacts specifically with the NH2 terminus of atrophin-1 (Fig. 1 B). There was no discernible difference in the strength of the interaction of ETO/MTG8 with the 26 or 65 glutamine forms of atrophin-1.

To confirm that atrophin-1 and ETO/MTG8 coexist in physiologically relevant complexes, atrophin-1 was immunopurified from mouse brain using goat polyclonal anti-atrophin-1 antibody APG840 immobilized on a solid support. Immunoblotting with rabbit polyclonal anti-atrophin-1 antibody A142 (Wood et al., 1998) confirmed successful immunopurification of atrophin-1 (Fig. 1 C, left), while immunoblotting with rabbit polyclonal ETO/MTG8 antibody 2174 (Sach et al., 1998) demonstrated coimmunopurification of ETO/MTG8 with atrophin-1 (Fig. 1 C, right). Control immunopurification reactions were performed with goat IgG coupled to the same support matrix.

Atrophin-1 and ETO/MTG8 Colocalize in Discrete Nuclear Structures

To determine whether atrophin-1 and ETO/MTG8 colocalize in cells, full-length cDNA expression constructs for atrophin-1 and FLAG-ETO/MTG8 were cotransfected into Neuro-2a cells. Transfected cells were immunostained simultaneously with the anti-atrophin-1 rabbit polyclonal antibody A142 and the anti-FLAG M2 monoclonal antibody. A trophin-1 was observed in both a diffuse pattern of small nuclear dots (Fig. 2, a and c) and larger discrete nuclear structures (Fig. 2, b and d). DAPI counterstaining confirmed nuclear localization of these structures (results not shown). Confocal imaging microscopy clearly demonstrated colocalization of atrophin-1 and FLAG-ETO/MTG8 in many of the large discrete subnuclear structures (Fig. 2, b and d). Overlap of atrophin-1 and ETO/MTG8 in the small nuclear dots was also observed, but the diffuse nature of both signals makes it difficult to ascertain whether this represents true colocalization. One explanation for the observed variations in staining patterns between cells is variation in the expression levels between cells. A blinded observer (Table I) scored the percentage of transfected cells showing each pattern of staining or a combination of these staining patterns. A greater percentage of cells had the large nuclear bodies when ETO/MTG8 was cotransfected with atrophin-1 than when atrophin-1 was cotransfected with pCDNA3 containing no insert. This suggests that ETO/MTG8 helps recruit atrophin-1 to the large subnuclear structures. There were no significant differences between A t-FL-26Q and A t-FL-65Q transfected cells, or A t-FL-26Q plus ETO/MTG8 and A t-FL-65Q plus ETO/MTG8 transfected cells.
Immunohistochemical analysis of atrophin-1 transgenic and nontransgenic mouse brains with the anti–atrophin-1 antibody AP142 revealed the presence of structures similar to those illustrated in Fig. 2 in neuronal nuclei (Schilling et al., 1999). However, we have been unable to observe a similar pattern of nuclear staining in mouse brain sections using the antibodies against ETO/MTG8 at our disposal. All of the anti-ETO/MTG8 antibodies tested showed high nonspecific background staining of brain sections and were deemed unsuitable for immunohistochemistry. Hence we have been unable to confirm that ETO/MTG8 is also localized to these structures in neurons.

ETO/MTG8 is a component of the N-CoR/mSin3/HDAC transcriptional repression complex (Gelmetti et al., 1998; Lutterbach et al., 1998b; Wang et al., 1998a). Consistent with this, the large nuclear structures observed in atrophin-1 and ETO/MTG8 cotransfected cells were labeled with antibodies to mSin3A, HDAC1, and HDAC2 (Fig. 3a, data not shown, and Fig. 3b, respectively). However, these structures were not labeled with an antibody to N-CoR (results not shown) and immunoblotting of Neuro-2a extracts failed to detect N-CoR in these cells (results not shown).

The large atrophin-1- and ETO/MTG8-containing structures are similar in size and number to PML oncogenic domains (PODs). A typical mammalian cell contains ~10–20 PODs, which are ~0.3–1 μm in diameter (Lamond and Earnshaw, 1998). However, the atrophin-1 and ETO/MTG8 immunoreactive nuclear structures were not labeled with antibodies to the PML, suggesting that they are distinct from PODs. Labeling for endogenous murine PML was not observed in Neuro-2a cells. To confirm that these structures were not PODs, Neuro-2a cells were cotransfected with atrophin-1, FLAG-ETO/MTG8, and human PML expression constructs, then immunostained for PML and atrophin-1 or PML and FLAG. No colocalization of PML with either atrophin-1 or FLAG-ETO/MTG8 was observed, and atrophin-1–expressing cells appeared not to contain PODs. Instead, PML was localized to either the nuclear periphery or to one or two very large nuclear structures, which may represent coalescence of PODs (Fig. 3, c and d).

Figure 1. Atrophin-1 interacts with ETO/MTG8. (A) Summary of data obtained from yeast two-hybrid screening. The degree of homology between the proteins is illustrated between the schematics. Lines represent clones isolated by yeast two-hybrid screening of a human cerebellum library with pPC97-A in T2-455. P/S/T, proline/threonine-rich domain; TAF110, region with homology to yeast transcription activating factor 110; Pro, proline-rich region; HHR, hydrophobic heptad repeat; Coil, coiled-coil domain; ZnF, zinc finger-like domains. (B) ETO/MTG8 interacts specifically with the NH2-terminal third of atrophin-1. No interaction was detected with the central portion of atrophin-1 containing the polyglutamine tract (with either 26 or 65 glutamines), the COOH-terminal half of atrophin-1, or the NH2-terminal 598 amino acids of huntingtin (with 18 or 81 glutamines). (C) Coimmunopurification of atrophin-1 and ETO/MTG8. A tropinin-1 protein complexes were purified with CarboLink-APG840 beads and immunoblotted for atrophin-1 (left) and ETO/MTG8 (right). Control immunoaffinity purifications were performed with CarboLink goat IgG.
been unable to test whether PML redistributes in neuronal nuclei showing atrophin-1 accumulation in DRPLA transgenic mice.

**Atrophin-1 Represses Transcription of a Reporter Gene**

ETO/MTG8 has been shown to bind to the repression domains of N-CoR and silencing mediator for retinoid and thyroid hormone receptors (SMRT; Gelmetti et al., 1998; Lutterbach et al., 1998b; Wang et al., 1998a) which form complexes with mSin3 and histone deacetylases. We observed colocalization of atrophin-1 with mSin3A, HDAC1, and HDAC2 in transfected Neuro-2a cells in both the presence (Fig. 3, a and b) and absence (results not shown) of cotransfected ETO/MTG8, leading us to hypothesize that atrophin-1 may itself be capable of influencing transcription.

GAL4 DBD–atrophin-1 fusion proteins repressed transcription of a luciferase reporter gene under the control of a thymidine kinase promoter downstream of four GAL4-binding sites in a dose-dependent manner in Neuro-2a cells (Fig. 4 a). Repression by the GAL4 DBD–atrophin-1 fusion proteins was enhanced by cotransfection with FLAG-ETO/MTG8 (Fig. 4 b), demonstrating a functional interaction between atrophin-1 and ETO/MTG8. Note that FLAG-ETO/MTG8 alone did not influence transcription of the luciferase reporter. The length of the glutamine repeat in atrophin-1 did not affect repression by GAL4 DBD–atrophin-1.

**Table I. Quantification of Atrophin-1 and ETO/MTG8 Nuclear Staining Patterns**

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Diffuse/small dots</th>
<th>Large structures</th>
<th>Combination</th>
</tr>
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<tbody>
<tr>
<td>At-FL-26Q only</td>
<td>28 ± 8</td>
<td>22 ± 2</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>At-FL-65Q only</td>
<td>32 ± 6</td>
<td>18 ± 2</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>ETO only</td>
<td>9 ± 3</td>
<td>42 ± 6</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>At-FL-26Q+ETO</td>
<td>6 ± 4</td>
<td>49 ± 3</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>At-FL-65Q+ETO</td>
<td>11 ± 6</td>
<td>52 ± 3</td>
<td>36 ± 3</td>
</tr>
</tbody>
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Cells were scored as having predominantly small dots or diffuse labeling (Fig. 2, a and c), predominately large structures (Fig. 2 d), or a combination of both staining patterns (Fig. 2 b).

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Figure 2. Atrophin-1 and ETO/MTG8 colocalize in large discrete subnuclear structures in cotransfected Neuro-2a cells. A t-26Q plus FLAG-ETO/MTG8 (a and b) and A t-65Q plus FLAG-ETO/MTG8 (c and d) cotransfected cells were double-labeled for atrophin-1 (green, left) and FLAG (red, center). The merged images are illustrated on the right.

Figure 3. A trophotin-1- and ETO/MTG8-containing nuclear structures stain for mSin3A and histone deacetylases, but not PML. (a and b) At-65Q and FLAG-ETO/MTG8 cotransfected Neuro-2a cells were stained for atrophin-1 (green, left) and mSin3A (red, center) and for atrophin-1 (red, left) and HDAC2 (green, center). The merged images (right) show that endogenous mSin3A and HDAC2 colocalize with transfected atrophin-1. (c and d) At-26/65Q, FLAG-ETO/MTG8 and PML cotransfected Neuro-2a cells were stained for atrophin-1 (green, left) and PML (red, center). The merged images (right) show that PML does not colocalize with atrophin-1. Cells expressing PML alone contained PODs, whereas cells coexpressing atrophin-1 did not appear to contain PODs; PML was localized to larger nuclear structures or the nuclear periphery in the presence of atrophin-1. Similar results were obtained by double labeling for FLAG and PML.
Atrophin-1– and ETO/MTG8-containing Nuclear Structures are SDS-soluble and Are Associated with the Nuclear Matrix

Expanded polyglutamine-containing proteins have an inherent ability to form SDS-insoluble aggregates (Scherzinger et al., 1997), and ubiquitinated neuronal intranuclear inclusions are a pathological hallmark of the polyglutamine neurodegenerative disorders. The large atrophin-1 immunoreactive bodies we observed were not stained with an antibody to ubiquitin (results not shown), and whole cell extracts were immunoblotted to confirm that the large atrophin-1 immunoreactive nuclear structures did not represent SDS-insoluble aggregates. ETO/MTG8 and atrophin-1 (with 26 or 65 glutamines) were completely solubilized in SDS-PAGE sample buffer, whether they were transfected alone or in combination (Fig. 5). There was no atrophin-1 or FLAG-ETO/MTG8 immunoreactive material at the top of the gel in any of the sample lanes. We routinely observe atrophin-1 immunoreactive material at the top of the gel when analyzing extracts from DRPLA transgenic mouse brains (Schilling et al., 1999). Hence we believe that the staining we observed represents a functional nuclear domain or structure, and not SDS-insoluble protein aggregates.

ETO/MTG8 has been shown to associate with the nuclear matrix (Chen et al., 1998; Le et al., 1998; McNiel et al., 1999). We hypothesized that the large nuclear structures containing atrophin-1 and ETO/MTG8 that we observed were associated with the nuclear matrix. To provide biochemical evidence for this hypothesis, nuclear subfractionation was performed (Fig. 6 A). When transfected alone into Neuro-2a cells, atrophin-1 was found to be predominantly associated with the Triton X-100 soluble fraction (Fig. 6 A, top two rows). A small proportion of atrophin-1 was detected in the nuclear matrix fraction of single-transfected cells. When atrophin-1 was cotransfected with ETO/MTG8, a much greater proportion of atrophin-1 was in the nuclear matrix fraction (Fig. 6 A, center two rows). Nearly all ETO/MTG8 partitioned into the nuclear matrix fractions of atrophin-1 and ETO/MTG8 cotransfected Neuro-2a cells (Fig. 6 A, bottom two rows). Therefore, expression of ETO/MTG8 recruited atrophin-1 to the nuclear matrix fraction.

To confirm that the observed nuclear structures are associated with the nuclear matrix, we performed immunoblotting.
cytochemical staining of nuclear matrix preparations in situ (Fig. 6 B). The large nuclear structures were associated with the matrix preparations and were clearly labeled with antibodies to atrophin-1 and FLAG-ETO/MTG8 (Fig. 6 B). The series of extractions performed stripped away most of the diffuse label seen after routine immuno-cytochemical staining (Figs. 2 and 3).

Atrophin-1 and ETO/MTG8 have both been shown to be expressed in the brain (Miyoshi et al., 1993; Nagafuchi et al., 1994; Davis et al., 1999), and be localized, at least in part, to neuronal nuclei (Yazawa et al., 1995; Sacchi et al., 1998; Schilling et al., 1999). We have previously shown that truncated fragments of atrophin-1 accumulate in neuronal nuclei in a transgenic mouse model of DRPLA (Schilling et al., 1999), while in this paper we have shown that atrophin-1 can be recruited to the nuclear matrix by ETO/MTG8. We reasoned that the fragments observed in DRPLA transgenic mice might also be associated with the nuclear matrix and we performed subfractionation of nuclei purified from DRPLA transgenic mice to test this hypothesis (Fig. 7). A atrophin-1 and ETO/MTG8 cofractionated almost exclusively with the insoluble nuclear matrix fraction. Both endogenous and transgene-derived atrophin-1 were associated with this fraction, and all transgene-derived species (full length, high molecular weight modified species, and truncated forms) associated with this fraction. A high molecular weight smear of atrophin-1 immunoreactivity was also apparent in the nuclear matrix preparation, suggesting that aggregated atrophin-1, possibly in inclusions, was also contained in this preparation.

ETO/MTG8 immunoreactivity was not detected at the top of the wells or in the stacking gel.

Discussion

Recent evidence suggests that DRPLA may be caused by the accumulation of truncated fragments of atrophin-1 in specific neuronal nuclei (Schilling et al., 1999). These truncated fragments contain the intact NH$_2$ terminus of atrophin-1 (Wood, J.D., unpublished observations). To date, the function(s) of atrophin-1 in the nucleus are unknown. We used the yeast two-hybrid system to find proteins that interact with the NH$_2$ terminus of atrophin-1 and identified ETO/MTG8, a protein known to be associated with transcriptional corepressor complexes. ETO/MTG8 coimmunopurified with atrophin-1 from brain extracts, suggesting that the interaction is physiologically relevant. When cotransfected into Neuro-2a cells, atrophin-1 and ETO/MTG8 colocalized in discrete nuclear matrix-associated structures, which immunostained for the endogenous corepressor complex proteins mSin3A, HDAC1, and HDAC2. Furthermore, atrophin-1 and ETO/MTG8 cofractionated in the nuclear matrix fraction of mouse brain nuclei. We also demonstrated that atrophin-1 can repress transcription in a reporter gene model system, and that repression was enhanced by cotransfection with ETO/MTG8. Together, these findings show that atrophin-1 can be a component of transcriptional corepressor complexes and may...
be involved in the regulation of gene transcription in the nucleus. Hence the accumulation of truncated atrophin-1 fragments in specific neuronal nuclei in DRPLA could interfere with transcriptional control and contribute to the disease phenotype.

ETO/MTG8 was identified as the fusion partner of AML1 in t(8;21) translocations causing acute myeloid leukemia (Erickson et al., 1992; Nisson et al., 1992; Miyoshi et al., 1993). ETO/MTG8 contains three proline-rich or proline/serine/threonine-rich regions, the COOH-terminal one of which contains a PEST sequence (Rechsteiner and Rogers, 1996), a signal for degradation by the ubiquitin-proteasome pathway. It also contains two zinc finger-like motifs (alternatively referred to as the MYND motif; Lutterbach et al., 1998a) which interact with N-CoR (Gelmetti et al., 1998b; Lutterbach et al., 1998b; Wang et al., 1998a). N-CoR is one member of a family of related proteins, including SMRT, and ETO/MTG8 has been shown to bind to SMRT and mSin3 independently of its interaction with N-CoR (Gelmetti et al., 1998b; Lutterbach et al., 1998b). This may explain why we were able to observe complexes of atrophin-1, ETO/MTG8, mSin3A, and histone deacetylases in the apparent absence of N-CoR.

ETO/MTG8 is one member of a family of highly conserved proteins. The other human family members identified to date are MTGR1 (Kitabayashi et al., 1998b) and MTG16 (Gamou et al., 1998). We isolated a single clone for MTG16 in our initial two-hybrid screen. Messenger RNA for ETO/MTG8 is relatively abundant in brain, testis, and ovary, present at a lower level in heart and lung, and essentially absent in other tissues examined to date (Davis et al., 1998; Miyoshi et al., 1993). The expression patterns of MTGR1 and MTG16 are more widespread (Gamou et al., 1998; Kitabayashi et al., 1998). ETO/MTG8, MTGR1, and MTG16 can self-associate and associate with each other via a hydrophobic repeat peptide (Davis et al., 1998; Kitabayashi et al., 1998; Lutterbach et al., 1998a), suggesting that they may coexist in transcriptional regulatory complexes.

An emerging link between the disease-associated polyglutamine proteins is nuclear matrix association. A taxin-1 and ataxin-3 both have been shown to be associated with the nuclear matrix (Skinner et al., 1997; Tait et al., 1998). A taxin-1 interacts in a polyglutamine tract-length-dependent manner with the cerebellar leucine-rich acidic nuclear protein in discrete nuclear matrix-associated structures (Maitilla et al., 1997). Here we demonstrate that both normal (unexpanded) and various forms of mutant atrophin-1 accumulate in DRPLA transgenic mouse brains. Nuclei were isolated from 6-mo-old AT-FL-65Q-150 DRPLA transgenic mice then extracted/digested sequentially with 0.5% Triton X-100, 250 mM (NH₄)₂SO₄, and 300 U/ml DNase I. The supernatants from these treatments and the insoluble matrix fraction were immunoblotted for atrophin-1 (antibody AP142, top) and ETO/MTG8 (antibody 2174, bottom). The majority of the immunoreactivity for both proteins was associated with the matrix preparation. The atrophin-1 blot shows a triplet of bands at around 190–220 kD. The faint lower band (bottom arrow) corresponds to full-length endogenous atrophin-1, the middle band (center arrow) corresponds to full-length transgene-derived mutant atrophin-1, and the upper band (top arrow) is an expanded polyglutamine-specific modified form of atrophin-1. The identity of these bands was ascertained by comparing the banding patterns in extracts from nontransgenic mice, DRPLA transgenic mice, human DRPLA cerebella, and human control cerebella. Other high molecular weight species and truncated forms of atrophin-1 (the predominate band at ~120 kD) were also associated with the matrix preparation.

Figure 7. A trophic-1 and ETO/MTG8 cofractionate with the nuclear matrix fraction of DRPLA transgenic mouse brains. Nuclei were isolated from 6-mo-old AT-FL-65Q-150 DRPLA transgenic mice then extracted/digested sequentially with 0.5% Triton X-100, 250 mM (NH₄)₂SO₄, and 300 U/ml DNase I. The supernatants from these treatments and the insoluble matrix fraction were immunoblotted for atrophin-1 (antibody AP142, top) and ETO/MTG8 (antibody 2174, bottom). The majority of the immunoreactivity for both proteins was associated with the matrix preparation.
The gene products for five, possibly six, genes mutated in polyglutamine neurodegenerative disorders can now be linked to transcriptional control via nuclear receptor signaling. The androgen receptor is a DNA-binding transcription factor that contains an unstable polyglutamine tract that is expanded in patients with X-linked spinal and bulbar muscular atrophy (SBMA) or Kennedy’s disease (L. Spada et al., 1991). Here we show that atrophin-1, the DRPLA gene product, represses transcription and interacts with ETO/MTG8 in nuclear-matrix-associated structures that contain mSin3 and histone deacetylases. ETO/MTG8 interacts with N-CoR, while N-CoR interacts with huntingtin, the Huntington’s disease gene product, in a polyglutamine repeat-length-dependent manner (Boutell et al., 1999).

Nuclear receptors such as the retinoic acid receptor (RA Rxα) and retinoid X receptor (RXR α) can associate with PODs. The function of PODs remains enigmatic, although they may be related to gene transcription and cell death (Quignon et al., 1998; Wang et al., 1998b). The spinocerebellar ataxia type 1 and 3 gene products have both been linked to PODs: mutant ataxin-1 redistributes PML and colocalizes with PODs to some extent, whereas mutant ataxin-3 aggregates colocalize with PODs (Chai et al., 1999; Skinner et al., 1997). We have found that atrophin-1– and ETO/MTG8-containing nuclear complexes appear to be distinct from PODs, but overexpression of atrophin-1 and ETO/MTG8 with PML led to apparent redistribution of PML. Unlike with ataxin-1, PML redistribution was independent of the length of the glutamine repeat in atrophin-1. It should also be noted that expansion of the polyglutamine tract in the TATA-binding protein, a basal transcription factor, may cause neurological disease (Koido et al., 1989).

It is apparent that the normal function of a number of the polyglutamine disease proteins may include transcriptional regulation, and there is evidence that altered gene transcription may be a major cause of cellular dysfunction in these diseases. Huntington’s disease transgenic mice show alterations in the levels of mRNA for components of neurotransmitter, calcium, and retinoid signaling pathways at early and late symptomatic time points (Cha et al., 1998; Luthi-Carter et al., 2000), suggesting that transcriptional regulatory mechanisms are affected in the brains of these mice. Such effects are not restricted to Huntington’s disease as polyglutamine expansion in ataxin-1 downregulates specific neuronal genes before pathologic changes occur in SCA 1 mice (Lin et al., 2000). Part of each disease phenotype could reflect interference with the normal function of each polyglutamine protein. However, all of the polyglutamine diseases show autosomal dominant inheritance, suggesting that these diseases are caused by novel gains of function of the expanded polyglutamine proteins, and not simply losses of function. Consistent with such a mechanism, we have found that mutant expanded huntingtin and atrophin-1, but not the normal unexpanded proteins, sequester CBP and interfere with CBP-mediated transcription, which adversely affects neuronal survival (Nucifora, F.C., Jr., M. Sasaki, M.F. Peters, H. Huang, J. Troncoso, V.L. Dawson, T.M. Dawson, and C.A. Ross, manuscript submitted for publication). This type of mechanism could be common to the polyglutamine disorders and distinct from the normal transcriptional role of each protein.

In summary, we have shown that atrophin-1 interacts with ETO/MTG8, that atrophin-1– and ETO/MTG8-containing complexes are associated with the nuclear matrix, and that atrophin-1 can repress transcription. These findings shed considerable new light on the function of atrophin-1 in the nucleus and may also be relevant to pathologic neuronal dysfunction in DRPLA. The emerging links between the neurodegenerative disease–associated polyglutamine proteins, translocation-leukemia proteins, nuclear receptor corepressor complexes, and the nuclear matrix may have wider repercussions for the whole family of polyglutamine diseases.

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