

Spinocerebellar ataxia type-1 and spinobulbar muscular atrophy gene products interact with glyceraldehyde-3-phosphate dehydrogenase

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Spinocerebellar ataxia type1 (SCA1) is one of several neurodegenerative disorders caused by expansions of translated CAG trinucleotide repeats which code for polyglutamine in the respective proteins. Most hypotheses about the molecular defect in these disorders suggest a gain of function, which may involve interactions with other proteins via the expanded polyglutamine tract. In this study we used ataxin-1, the SCA1 gene product, as a bait in the yeast two-hybrid system and identified the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase as an ataxin-1 interacting protein. In addition, the yeast two hybrid data demonstrate that wild type and mutant ataxin-1 form homo and heterodimers. Physical interaction between GAPDH and ataxin-1 was also demonstrated *in vitro*. To investigate if GAPDH might interact with other glutamine repeat-containing proteins involved in neurodegenerative disorders, we tested its binding to the androgen receptor which is mutated in spinobulbar muscular atrophy. The androgen receptor interacts with GAPDH both in the yeast two-hybrid system and *in vitro*. The binding of both ataxin-1 and the androgen receptor to GAPDH does not vary with the length of the polyglutamine tract. While provocative, these findings do not address the selective neuronal loss in each of these disorders in light of the wide expression patterns of GAPDH and the respective polyglutamine containing proteins. Nonetheless, such interactions may increase the susceptibility of specific neurons to a variety of insults and initiate degeneration.

INTRODUCTION

To date, five neurodegenerative disorders have been shown to be caused by expansions of CAG repeats which lie within the coding regions of their respective genes. These include spinobulbar

muscular atrophy (SBMA), Huntington disease (HD), spinocerebellar ataxia type-1 (SCA1), dentatorubropallidoluysian atrophy (DRPLA), and Machado-Joseph disease (MJD) (1). The CAG repeat codes for a glutamine tract in each of the involved proteins and the length of the tract correlates inversely with the age of onset and severity of disease (2,3). In SCA1, normal alleles have a repeat number ranging from 6–44 usually interrupted with one or more CAT triplets when the CAG tract is longer than 21 repeats, while the SCA1 alleles have an unstable uninterrupted CAG repeat which ranges from 40–82 (4,5). The proteins implicated in each of these diseases are dissimilar except for the polymorphic polyglutamine tracts, and for most the normal function is unknown (2). In all five disorders, the transcripts for the respective genes are widely expressed while the neurodegeneration is cell-specific. The molecular mechanism by which an expanded polyglutamine tract leads to cell-specific neurodegeneration remains unknown.

Numerous observations argue against a loss of function mechanism in the pathogenesis of CAG-mediated neurodegenerative diseases. These include the absence of a HD-like phenotype in heterozygous individuals with deletions of the HD gene and the lack of motor neuron disease in males with deletions of the androgen receptor (AR), the gene implicated in SBMA (6–8). Furthermore, gene targeting of the HD gene in mice leads to embryonic lethality in null mutant mice and apparently normal or subtle neurobehavioral phenotype in heterozygotes with no evidence of HD neuropathology (9–11). The finding that in each of the mutated genes, the CAG repeats are found in the coding region and in each case encode a polyglutamine tract, suggests that this mutation exerts its effects at the protein level. Furthermore, transgenic mice carrying a human SCA1 cDNA with an expanded number of CAG repeats under the control of a Purkinje cell specific promoter develop ataxia and Purkinje cell degeneration (12). In SCA1 transgenic mice, the mutant SCA1 protein was demonstrated to be translatable and although it was not detected by western analysis, it was found at high levels in Purkinje cells by immunohistochemistry (Zoghbi and Orr, unpublished data). These data support the hypothesis that a gain of novel properties by the product of the expanded allele is

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responsible for the pathogenesis, at least in SCA1. The gain of function may be mediated by specific protein-protein interactions involving the expanded glutamine repeat and possibly a related set of molecules, which thus far remain unidentified.

To identify proteins that interact with the SCA1 gene product, we used the yeast two-hybrid system of interaction cloning (13,14). In this study, we report the identification of a glycolytic enzyme, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an ataxin-1 interacting protein. Furthermore, we demonstrate that GAPDH also interacts with the androgen receptor, the gene product mutated in SBMA. The interaction of both ataxin-1 and the androgen receptor with GAPDH suggests that in SCA1 and SBMA, the onset of neuronal cell death may share common biochemical mechanisms and may in part be mediated through interaction with a common set of cellular proteins.

RESULTS

Isolation of ataxin-1 interacting proteins

For the yeast two-hybrid screen, we used a fusion protein bait consisting of the yeast Gal4 DNA-binding domain and the ataxin-1 protein (amino acids 1–819) expressed from a yeast shuttle vector pAS2-CYH2 (see Materials and Methods). This bait plasmid was co-transformed into the yeast strain Y190 with a mouse embryonic cDNA expression library (10.5 days *post coitum*) fused to the Gal4 activation domain in the vector pACT2 (15). The mouse embryonic library was selected for identifying ataxin-1 interacting proteins because it is a high quality library and at this time there are no high quality human cerebellar libraries from either fetal or adult tissue available in the appropriate vectors for the two hybrid screen. Furthermore, we rationalized that there is no reason to assume *a priori* that the putative interactor will be expressed only in adult tissues. About 10^5 transformants were screened for activation of the *HIS3* and *LacZ* (β -galactosidase) reporter genes. After the isolation of *Trp*⁺, *Leu*⁺, *His*⁺, *LacZ*⁺ colonies, a chemical selection on cycloheximide media resulted in a strain that is devoid of the bait plasmid but carries the library plasmid (15). This assay eliminates putative interacting proteins which activate the reporter genes in the absence of the bait, ataxin-1. One of the positive clones, clone II.1 was identified as the glyceraldehyde-3-phosphate dehydrogenase gene (amino acids 1–170) by sequence analysis. To determine the specificity of the interaction, control experiments with dihybrid crosses showed that GAPDH specifically interacted with ataxin-1; matings of yeast containing the GAPDH clone with those expressing a battery of heterologous bait fusion proteins formed diploids that showed no detectable β -galactosidase activity. In contrast, the Gal4-ataxin-1 strain exhibited strong β -galactosidase activity when mated to yeast carrying either the library GAPDH clone (Fig. 1) or the full length rat GAPDH fused to the Gal4 activation domain in pGAD424 (data not shown). These experiments indicate that the interaction produced by these clones in the two-hybrid system is specific for ataxin-1 and is not due to the promiscuous interaction of GAPDH with random cellular proteins. Using the two-hybrid system we were also able to show that ataxin-1 self-associates (Fig. 1) and that the association occurs between wild type molecules or mutant molecules (homodimerization) as well as between wild type and mutant gene products (heterodimerization).

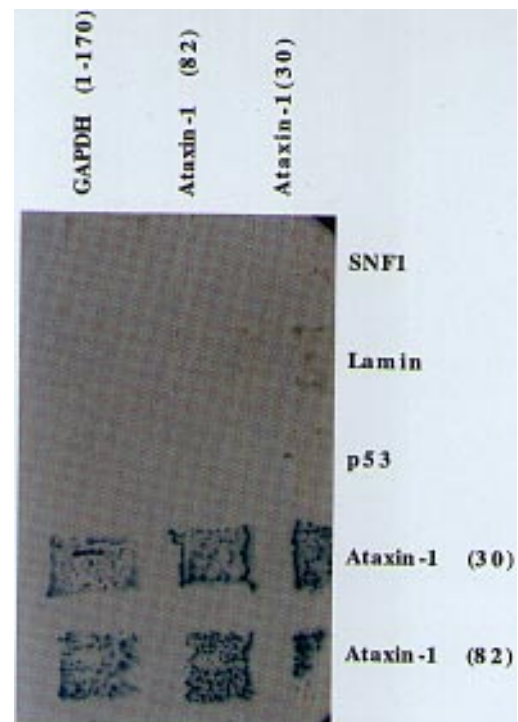


Figure 1. Specificity of the interaction of ataxin-1 with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in yeast. Yeast strain Y187 containing the cDNA clone encoding amino acids 1–170 of GAPDH fused to the Gal4 activation domain in the pACT2 vector was mated with yeast strain Y190 (MAT α X MAT α) carrying pAS2 plasmids expressing either wild type (30 repeats) or mutant ataxin-1 (82 repeats), or heterologous bait proteins, lamin, SNF1, and p53 to form diploids. Interactions of either wild type or mutant ataxin-1 with GAPDH formed diploids that gave a strong blue color on X-gal staining. All other diploids were negative for β -galactosidase activity.

N-terminal polyglutamine containing domain of ataxin-1 interacts with the dinucleotide binding domain of GAPDH

To delineate the region of the ataxin-1 protein that interacts with GAPDH, a set of constructs containing N-terminal and C-terminal deletions were created using the polymerase chain reaction, cloned into the pAS2-CYH2 plasmid and used to co-transform the HF7c strain which expresses the GAPDH clone. The pattern of interaction of ataxin-1 deletion mutants with GAPDH is summarized in Figure 2. Ataxin-1 was found to interact with GAPDH through the N-terminal domain containing the polyglutamine stretch. In addition, clone Pre-N which contains the N-terminal portion of the protein minus the glutamine repeats showed binding to GAPDH albeit at a lower level than the clone N-N', which also contains the glutamine repeat region. This suggests that there is some specificity of binding of GAPDH to ataxin-1. None of the clones which contained only the carboxy terminal regions of ataxin-1 showed interaction with the enzyme. These results identify the N-terminal portion (amino acids 1–300) of ataxin-1 as critical for interaction with GAPDH.

Analysis of the sequence of the library GAPDH clone revealed that it contained the nicotinamide adenine dinucleotide (NAD) binding domain (amino acids 1–149) and the first 21 residues

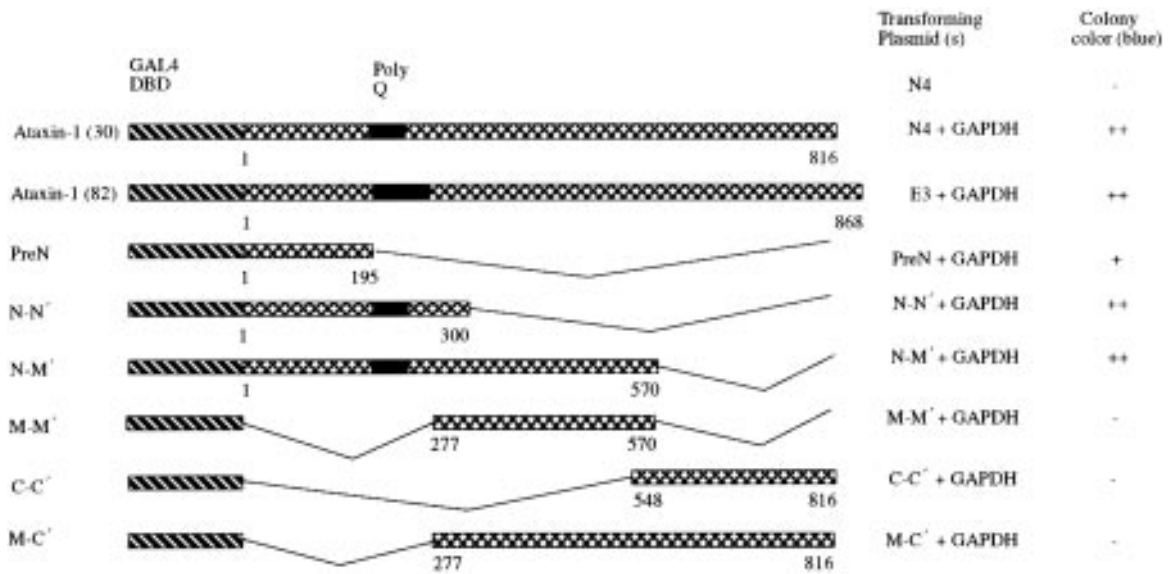


Figure 2. Ataxin-1 binds GAPDH through its amino terminal region containing the glutamine repeats. Schematic of Gal4-ataxin-1 fusion proteins used to determine the binding domains. The Gal4 DNA binding domain (DBD, amino acids 1–147; hatched box) is fused to the various ataxin-1 constructs in the pAS2-CYH vector. The glutamine repeat domain (Poly Q) is shown as filled boxes. N4 refers to the wild type ataxin-1 (30 repeats) pAS2 construct and E3 to the mutant ataxin-1 (82 repeats) pAS2 construct. Interactions between GAPDH and the various ataxin-1 constructs was determined after co-transformation in the yeast strain HF7c. Color of the colonies was assessed by the colony lift assay and indicated as (+) present, (–) absent and (++) for a more intense blue color.

(amino acids 149–170) from the catalytic domain (amino acids 149–312) including the active site cysteine (Cys-149) of the GAPDH enzyme (16–18). Because only a small portion of the catalytic domain is present in the interacting GAPDH subclone (21 out of 163 amino acids), it can be inferred that the site of interaction of ataxin-1 with the GAPDH enzyme is likely the NAD binding site. Full length rat GAPDH cDNA when cloned in pGAD424 also interacted with ataxin-1.

In vitro association of GAPDH and ataxin-1

To confirm the interaction observed in yeast, we examined whether GAPDH and ataxin-1 associate *in vitro*. Rabbit muscle GAPDH was immobilized on Affigel and incubated with either wild type ataxin-1 (32 repeats) or mutant ataxin-1 (82 repeats) expressed in COS7 cells. Following extensive washing, bound proteins were run on an SDS-polyacrylamide gel, blotted and probed with an anti-ataxin-1 antibody. As seen in Figure 3a, both the wild type and mutant ataxin-1 proteins were specifically retained on the GAPDH beads. As a control, we used Affigel beads whose active groups had been blocked. No retention of either forms of ataxin-1 was observed using the beads alone (Fig. 3b). The strength of the binding interaction was assessed using a gradient of NaCl washes. The binding of ataxin-1 to GAPDH persisted up to a 1 M NaCl wash (Fig. 4). Similar results were obtained when using COS7 cells expressing mutant ataxin-1 (data not shown). Elution of ataxin-1 from the GAPDH resin was only possible with 2% Formic acid (data not shown).

To investigate the binding of ataxin-1 with varying numbers of glutamines to GAPDH we used protein extracts from lymphoblastoid cell lines of SCA1 patients with different CAG repeat lengths. Ataxin-1 alleles containing 30, 43 and 60 glutamines

were retained on the GAPDH resin (Fig. 5). We did not see any discernible differences in the binding of the various allele sizes as ascertained by SDS-PAGE and autoradiography. Ataxin-1 with varying repeat sizes also did not show differential elution patterns with varying concentrations of NaCl. These experiments further establish the interaction of ataxin-1 and GAPDH *in vitro*.

Interaction of the androgen receptor with GAPDH

To determine if binding of ataxin-1 to GAPDH was unique or if it was a common feature of other glutamine repeat containing proteins involved in neurodegenerative disorders, we investigated the binding of the androgen receptor (AR) implicated in SBMA to GAPDH. First, we used the yeast two-hybrid system to demonstrate AR-GAPDH interaction. Amino terminal portions of the androgen receptor containing 24 (amino acids 13–134), 45 (amino acids 13–155) and 66 (amino acids 13–176) glutamines were cloned in pAS2 (Clontech) and transformed into the yeast strain Y187 for mating analysis. This yeast strain was then mated to HF7c which carries the GAPDH clone (amino acids 1–170 fused to the Gal4 DNA activation domain) or pGAD424 which carries the full length rat GAPDH cDNA. The resulting diploids were assayed for β -galactosidase activity. Figure 6a, shows that all three alleles of the androgen receptor interact with GAPDH. To confirm this interaction, full length androgen receptor constructs with 24, 45 and 66 CAG repeats were expressed in COS7 cells and protein extracts from these cells were incubated with GAPDH linked to the Affigel resin. Figure 6b shows that all three androgen receptor proteins with varying repeat lengths are retained on the GAPDH resin. These results demonstrate that binding to GAPDH is not a phenomenon that is restricted to

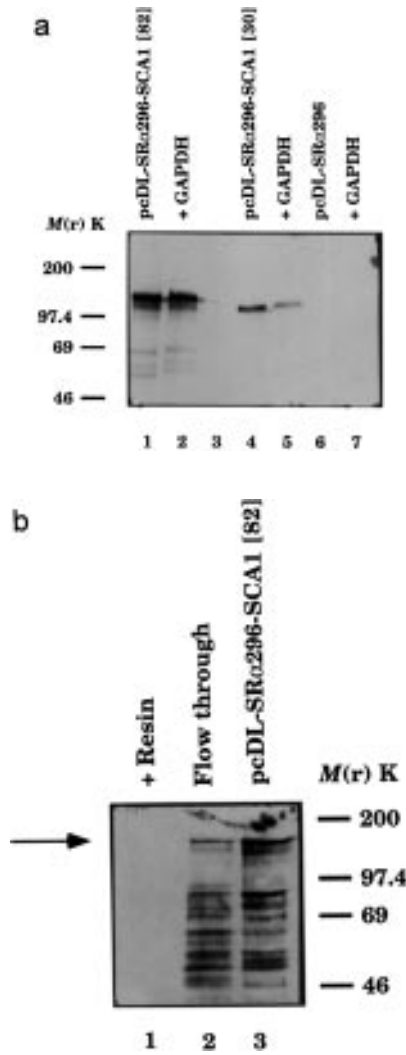


Figure 3. *In vitro* binding of GAPDH and ataxin-1. (a) COS7 cell extracts expressing either full length mutant ataxin-1, 82 repeats [pcDL-SR α 296-SCA1 (82)] in lane 1 or wild type ataxin-1, 30 repeats [pcDL-SR α 296-SCA1 (30)] in lane 4 were incubated with rabbit muscle GAPDH coupled to Affigel-10 (lanes 2 and 5 respectively). Following washing, complexes were analyzed by SDS-PAGE and immunoblotting using an anti-ataxin-1 antibody, 11750V. Lanes 2 and 5 show that ataxin-1 is retained on the GAPDH resin. Lanes 6 and 7 contain extracts from COS7 cells transfected with vector alone before and after binding to GAPDH beads. Lane 3 is empty. (b) The result of a control experiment carried out using the COS7 cell extract expressing the mutant ataxin-1 protein incubated with the Affigel-10 resin not coupled to any protein. No retention of mutant ataxin-1 with the resin alone was detected in lane 1. Immunoreactivity for ataxin-1 marked by an arrow is detected in the flow through and the COS7 extract, lanes 2 and 3.

ataxin-1 but possibly is a feature of the proteins involved in other glutamine repeat disorders.

β -galactosidase assay for GAPDH-ataxin-1 and GAPDH-AR interactions

To determine if the length of the glutamine repeat tract influenced the strength of the binding interaction between either ataxin-1 or the androgen receptor with the GAPDH protein, all interactions were assessed by a quantitative *o*-nitrophenyl- β -D-galactopyranoside based liquid assay. Although this assay is not a true

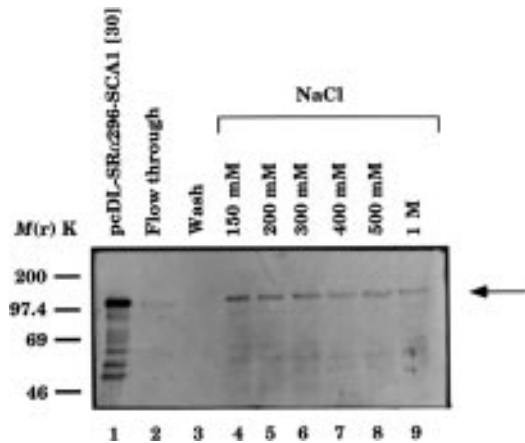


Figure 4. Binding of GAPDH to ataxin-1 persists after high salt washes. Wild type ataxin-1 expressed in COS7 cells [pcDL-SR α 296-SCA1 (30)] was incubated with GAPDH bound to Affigel-10. Lane 1 contains COS7 extract, lane 2, the flow through after binding to GAPDH; lane 3 contains the first wash; lanes 4-9 contain GAPDH-ataxin-1 complexes after serial washes with increasing molar concentrations of NaCl. The arrow points to the ataxin-1 band. Ataxin-1 did not elute from the GAPDH-coupled resin even at a concentration of 1 M NaCl.

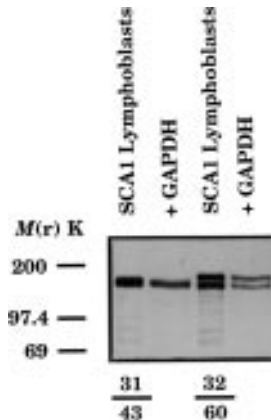


Figure 5. Binding of various ataxin-1 alleles from lymphoblast extracts of SCA1 individuals to GAPDH. The number of the CAG repeats in the alleles is shown at the bottom of the panel. Both normal as well as expanded alleles are retained on the GAPDH resin (lanes marked + GAPDH).

assessment of actual affinities in mammalian cells, it reflects trends in the strengths of the interaction. Liquid cultures of yeast Y190 or HF7c transformed with either ataxin-1 (30 or 82 repeats) or the androgen receptor (24, 45 or 66 repeats) and GAPDH were grown and assayed for β -galactosidase activity.

β -galactosidase activity after co-transformation of GAPDH with ataxin-1 was similar for the wild type (mean specific activity \pm standard deviation = 4.2 ± 0.80) and mutant protein (3.7 ± 0.82); $P = 0.59$ (Fig. 7a). Similarly, the androgen receptor with the largest number of repeats did not have a higher binding strength to GAPDH. Specific β -galactosidase activity was 4.6 ± 2.5 , 6.8 ± 0.34 , 7.9 ± 1.9 in case of the GAPDH interaction with AR24, AR45 and AR66, respectively; $P = 0.59$ (Fig. 7b). These results indicate that the length of the glutamine tract in ataxin-1 and the androgen receptor does not significantly affect the strength of the binding to GAPDH as measured using β -galactosidase assay in yeast.

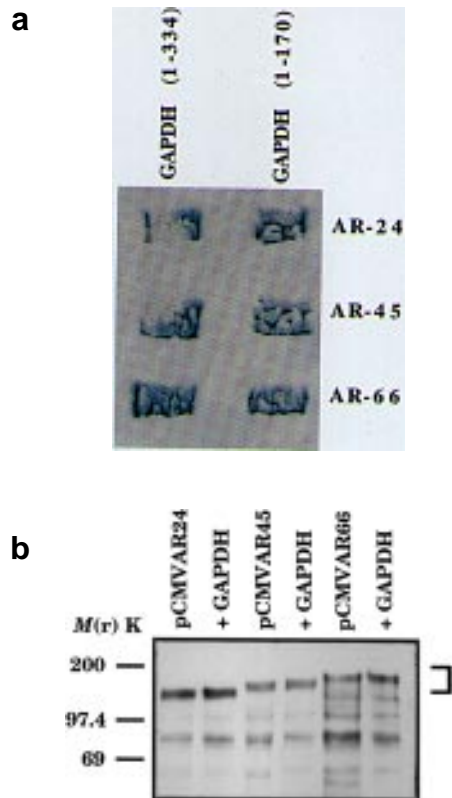


Figure 6. (a) Interaction of the androgen receptor containing various numbers of glutamines (24, 45 or 66) with GAPDH in yeast using the mating assay. The nomenclature of the clones containing the AR-Gal4 DNA binding domain fusion proteins identifies the number of repeats in each clone; other vectors carrying the GAPDH clones and the yeast strains are as discussed in legend to Figure 1. Diploids were screened for β -galactosidase activity. All of the AR alleles interact with GAPDH *in vivo* in yeast. (b) *In vitro* binding of AR proteins containing 24, 45 and 66 glutamines respectively to GAPDH. AR proteins expressed in COS7 cells were incubated with GAPDH coupled to Affigel-10 and washed extensively. Complexes were analyzed by immunoblotting using a mAb to the androgen receptor, F39.4.1. Brackets indicate the position of the AR protein before and after binding to to GAPDH (+ GAPDH lanes).

DISCUSSION

We identified the glycolytic enzyme GAPDH as a protein that interacts with both ataxin-1 and the androgen receptor. Furthermore, we used different approaches to demonstrate that ataxin-1 and the androgen receptor interact specifically with GAPDH. When assayed in yeast, GAPDH interacts with ataxin-1 and the androgen receptor but not with a number of other heterologous bait proteins. Analysis of deletion constructs localized the portion of ataxin-1 that binds to GAPDH to its amino terminal region containing the polyglutamine tract. Binding studies show that GAPDH also interacts with the two proteins *in vitro*, confirming the results of the yeast interaction assay. The binding between both wild type and mutant ataxin-1 molecules could not be disrupted by up to a 1 M NaCl wash. This strong binding is atypical of most protein-protein interactions which are generally disassociated by salt concentrations below 1 M and suggests that both forms of ataxin-1 have a very strong binding interaction with GAPDH. Recently, it has been shown that this ubiquitous enzyme also interacts with the gene products from two other glutamine repeat disorders, huntingtin and the DRPLA protein (19). Using the liquid β -galactosidase assay, increased repeat sizes do not have a significant effect on the binding interaction between GAPDH and the ataxin-1 or androgen receptor proteins. Our results differ from those reported by Burke *et al.* (19) who indicate using polyglutamine affinity columns that GAPDH binds only to the 60-glutamine peptide and not to the 20-glutamine peptide. The reasons for the possible differences in the results could be related to the fact that we tested for length dependency *in vivo* using the yeast two-hybrid system with full length ataxin-1 containing either 30 or 82 glutamines, whereas Burke *et al.* used immobilized polyglutamine peptides. In addition, our *in vitro* binding experiments also indicate that full length wild type and mutant ataxin-1 proteins from SCA1 patient lymphoblast extracts do not show differences in their binding pattern to immobilized GAPDH.

It has been proposed that a gain of function mechanism involving the mutant protein with expanded polyglutamine tract caused by CAG repeat expansions is the molecular basis of the selective neuronal loss seen in all the disorders (2). This novel gain of function may be due to aberrant interactions with other

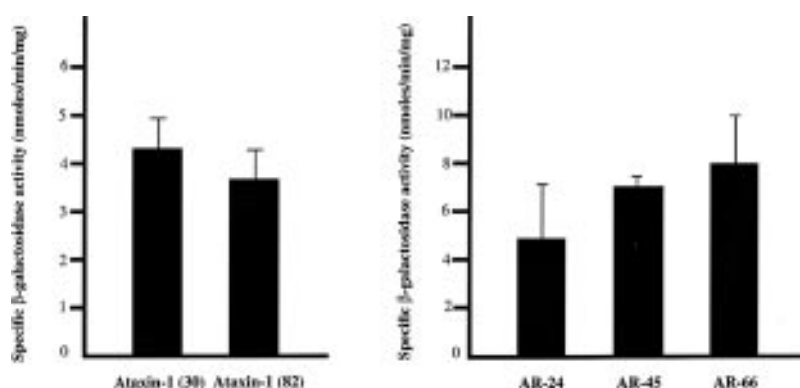


Figure 7. Quantitation of β -galactosidase activity in yeast transformed with GAPDH clone and wild type ataxin-1, mutant ataxin-1 or the androgen receptor (24, 45 or 66 repeats). β -galactosidase activity is indicated as nanomoles per minute per milligram protein (mean \pm standard deviation) on the Y-axis. On the X-axis, the clone that was used to assess interaction with the GAPDH is indicated.

cellular proteins. The binding of GAPDH to both ataxin-1 and AR raises a question about the biological significance of this interaction *vis-à-vis* neurodegeneration. GAPDH is a regulatory enzyme in glycolysis which catalyzes the oxidation and phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and is involved in the glycolytic conversion of glucose to pyruvic acid (20,21). In addition, GAPDH has also been implicated in an array of cellular functions that are seemingly independent of its role in glycolysis. These include interaction with microtubules (22), tRNA (23), the C-terminal region of the amyloid precursor protein (APP) (24,25), involvement in DNA repair (26,27), and protein phosphorylation (28). Although GAPDH is historically known as a cytoplasmic enzyme, several reports have demonstrated nuclear localization as well (23,29,30). It is possible that the binding of ataxin-1 or the androgen receptor to GAPDH may have deleterious effects on any of its functions, leading to neuronal degeneration, although at this time it is difficult to ascertain which of the functions of GAPDH are affected by the binding of the mutant ataxin-1 or androgen receptor.

Because ataxin-1 interacted with a partial GAPDH clone containing primarily the NAD binding domain (Rossmann Fold) and the first 21 residues from the catalytic domain, this binding could have critical effects on enzyme activity by either competing for the essential co-factor NAD or by obliterating its binding site on the enzyme (31). The fact that GAPDH is an abundant cellular protein whereas the levels of the glutamine-repeat containing proteins are remarkably lower in comparison raises questions about how a stoichiometric interaction between the two proteins is possible. The interaction of polyglutamine containing proteins with GAPDH could dissociate the enzyme from its active tetrameric form to inactive monomers or dimers since it is known that GAPDH exists in an equilibrium mixture of tetramers, dimers, and monomers (32) with the tetrameric form being responsible for the enzymatic activity (21). Although the etiology of neurodegenerative diseases remains enigmatic, it is compelling to propose that a defect in energy metabolism is the first event in the neurodegenerative cascade. The finding that the gene products from four different neurodegenerative disorders interact with GAPDH suggests that this protein may contribute to a common modality of pathogenesis. This argues in favor of the hypothesis that a slow decline in energy metabolism of a neuronal cell may trigger the degenerative process leading ultimately to cell death. The confinement of the degeneration to neuronal cells may be due to the fact that these cells are post-mitotic and do not proliferate like other peripheral cells. Hence unlike non-neuronal cells, they are more susceptible to chronic energy deprivation due to decreased ATP production from possibly partial loss of GAPDH activity. There is substantial evidence documenting impairments in energy metabolism which lead to excitotoxic neuronal injury and selective neuronal degeneration. For instance, inhibition of the succinate dehydrogenase by chronic administration of the inhibitor 3-nitro propionic acid produces selective striatal degeneration in primates and reproduces many of the clinical features of Huntington's disease (33,34). Similarly, cyanide poisoning which inactivates cytochrome oxidase results in selective degeneration of the globus pallidus and the putamen, regions which show neuronal loss in DRPLA (35). Therefore, it is possible that an impairment in energy metabolism can result in specific neuronal degeneration despite the wide expression pattern of GAPDH.

Alternatively, it is possible that the abundance and/or subcellular localization of the glutamine containing proteins varies with neuronal cell type and that it is this variability that accounts for the selective neurodegeneration. Lastly, the tissue-specificity possibly entails the interaction of a third protein that specifically binds the mutant form of the glutamine repeat proteins and whose expression is restricted to the tissues in which the phenotype is manifested. For instance, in Huntington disease, huntingtin binds to a novel protein, huntingtin-associated protein (HAP-1) which is selectively expressed in the brain. The binding between huntingtin and HAP-1 is dependent on the length of the polyglutamine tract (36). This type of tissue specific interaction will account for the selective neuronal loss and the final degeneration may be due to energy deprivation which is GAPDH dependent.

The available data from this study and that by Burke *et al.* (19) demonstrate that four glutamine-repeat containing proteins interact with GAPDH. This interaction may represent one component of a complex pathway that is likely to involve other protein(s) which account for the distinct clinical phenotypes and selective neuronal dysfunction.

MATERIALS AND METHODS

Plasmids and yeast strains

The plasmid vector pAS2-CYH2 used to construct the bait proteins has already been described (15). Full length wild type ataxin-1 cDNA with 30 repeats (pAS2-N4) or mutant containing 82 repeats (pAS2-E3) was cloned in frame in pAS2-CYH2 and their integrity confirmed by DNA sequencing. Similarly, cDNA coding for the N-terminal portions of the androgen receptor with 24, 45 and 66 repeats were cloned in the pAS2 vector (Clontech). Full length rat GAPDH was cloned in frame in the pGAD424 vector which contains the Gal4 activation domain. Yeast strains Y187, Y190 (15) and HF7c (37) have already been described.

Screening the cDNA library

Yeast strain Y190 was transformed with the bait plasmid pAS2-N4 and selected as previously described (14,38). The mouse embryonic library (10.5 d.p.c) tagged with the Gal4 activation domains in the pACT vector was obtained from Dr E. N. Olson. Y910 carrying the bait vector was transformed with the mouse embryonic library essentially as described (14). His⁺ colonies that were obtained were patched onto fresh plates and retested for the His⁺ phenotype and for β -galactosidase activity using the filter-lift method (39). Library plasmids from His⁺, β -gal⁺ colonies were recovered after growth of yeast on synthetic complete media plates lacking leucine (SC-Leu) plus cycloheximide 2.5 μ g/ml (15). Bait plasmid loss was confirmed by a Trp⁻ phenotype before plasmid DNA was recovered for transformation of *E. coli*. DNA sequences were determined directly from the pACT2 plasmid containing the cDNAs by automated sequencing. To assess the specificity of the interaction of ataxin-1 with GAPDH, Y187 carrying either the partial (amino acids 1–170 in pACT2) or full length GAPDH in pGAD424 was mated (MAT α X MAT α) with yeast strain Y190 carrying pAS2 plasmids expressing ataxin-1 (30 or 82 repeats) or heterologous bait proteins, lamin, SNF1, p53, cyclin D1, cdc 25, tat, cdk2, cdk6 to form diploids. In the diploids the association of expressed proteins results in the expression of the β -galactosidase reporter gene which was assayed using the filter lift method.

Expression vectors, protein preparation, and *in vitro* binding assays

COS7 cell extracts containing the wild type and mutant ataxin-1 proteins were prepared as previously described (12). Likewise, full length cDNAs for androgen receptor alleles containing 24, 45 and 66 repeats were cloned in pCMV5 vector, expressed in COS7 cells and cell extracts prepared (40). Extracts from lymphoblastoid cells from controls and SCA1 patients were prepared as described (3). *In vitro* binding assays were carried out as follows: Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Sigma) was coupled to Affigel-10 (BIO-RAD) according to the manufacturers instructions. As a control, Affigel-10 treated with triethanolamine to block reactive groups was used. Approximately 50 µg of total protein from COS7 cell extracts expressing either ataxin-1 alleles 30 or 82 repeats or the androgen receptor alleles 24, 45, and 66 glutamine repeats was used. In the case of the lymphoblastoid cell extracts from controls or SCA1 patients, 150 µg of total protein was used. Protein extracts were diluted into 300 µl of binding buffer [50 mM, HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, 1 mM PMSF, and 1 µg/ml each of leupeptin, pepstatin and aprotinin] and incubated with 250 µl of a 50% slurry of GAPDH coupled matrix for 1 h either at room temperature or 4°C. Following incubation, the resin was pelleted by centrifugation and the flow-through collected. The resin was then subjected to a series of washes, twice with 1 ml of binding buffer containing 0.05% Tween-20, followed by one wash each with 1 ml of 300 mM, 500 mM, and 1 M NaCl in binding buffer. After the last wash, the resin was centrifuged down and suspended in loading buffer and run on 10% SDS-PAGE gels. Proteins were detected by western blot analysis using antibody 11750V to ataxin-1 (3) and a mouse mAb F39.4.1 to the androgen receptor (41).

Quantitation of β-galactosidase activity in yeast

For quantitation of the strength of the interactions, yeast strains Y190 or HF7c transformed with the plasmids under study were grown to stationary phase in synthetic medium lacking leucine and tryptophan. Cultures were diluted 10-fold and regrown to a final A₆₀₀ of 0.8–1.2. β-galactosidase activity was determined as follows: 0.2 ml of yeast transformant extracts was added to 0.8 ml of Z buffer (60 mM Na₂HPO₄/40 mM NaH₂PO₄/10 mM KCl/1 mM MgSO₄, 40 mM 2-mercaptoethanol, pH 7.0) and warmed to 28°C. Reactions were initiated by addition of 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) in Z buffer and terminated using 0.5 ml of 1 M Na₂CO₃ and A₄₂₀ monitored. Values reported are the average of duplicate assays of five independent transformants. Specific β-galactosidase activity is expressed as nanomoles per minute per milligram protein (42). To determine if the size of the CAG repeat significantly affects the ataxin-1-GAPDH interaction, Levene's test, a homogeneity-of-variance test was employed. The non-parametric Kruskal-Wallis one-way ANOVA was used to evaluate the GAPDH-AR interaction. All values are represented as mean ± standard deviation.

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