A cell-based screen for modulators of ataxin-1 phosphorylation

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Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disorder caused by the expansion of a glutamine repeat within the SCA1-encoded protein ataxin-1. We have previously shown that serine 776 (S776) of both wild-type and mutant ataxin-1 is phosphorylated \textit{in vivo} and \textit{in vitro}. Moreover, preventing phosphorylation of this residue by replacing it with alanine resulted in a mutant protein, which was not pathogenic in spite of its nuclear localization. To further investigate pathways leading to S776 phosphorylation of ataxin-1, we developed a cell-culture based assay to screen for modulators of S776 phosphorylation. In this assay, ataxin-1 expression was monitored by enhanced green fluorescent protein (EGFP) fluorescence in cell lines stably expressing EGFP–ataxin-1 fusion protein. The phospho-S776 ataxin-1 specific antibody (PN1168) was used to assess ataxin-1 S776 phosphorylation. A library of 84 known kinase and phosphatase inhibitors was screened. Analysis of the list of drugs that modified S776 phosphorylation places many of the inhibited kinases into known cell signaling pathways. A pathway associated with calcium signaling resulted in phosphorylation of both wild-type and mutant ataxin-1. Interestingly, inhibitors of the PI3K/Akt pathway predominantly diminished mutant ataxin-1 phosphorylation. These results provide new molecular tools to aid in elucidating the biological role of ataxin-1 phosphorylation and perhaps provide potential leads toward the development of a therapy for SCA1.
Protein phosphorylation is a prominent mechanism to regulate protein folding, clearance and subcellular localization (10). Thus, the phosphorylation status of ataxin-1 was investigated and serine 776 (S776) was found to be phosphorylated both in vivo and in vitro (11). To further investigate the role of this phosphorylation event in vivo, the serine residue was mutated to alanine (A776), thus preventing its phosphorylation. Expression of ataxin-1[82Q]-A776 in transgenic mice resulted in a significant dampening of the pathogenic effects of mutant ataxin-1. On the basis of both behavioral analyses and Purkinje cell morphology, mutation of S776 to alanine rendered mutant ataxin-1 substantially less pathogenic. However, mutation of this residue did not affect the subcellular localization of mutant ataxin-1, indicating that phosphorylation of this residue is not required for nuclear transport. The serine to alanine mutation did result in a more slow form of mutant ataxin-1. In both tissue culture cells and transgenic mice, the frequency of inclusions was dramatically decreased. The finding that S776 was phosphorylated led to the identification of 14-3-3 as a protein, which interacts specifically with phospho-S776 ataxin-1 (12). 14-3-3 seemed to mediate the toxicity of mutant ataxin-1 by binding to and stabilizing the phospho-S776 mutant protein. Phosphorylation of ataxin-1 S776 by protein kinase B (Akt/PKB) regulated the association of 14-3-3 with mutant ataxin-1 (12). Akt/PKB is a component of the phosphatidylinositol 3-kinase signaling pathway, which controls a wide range of diverse cellular functions (13).

Protein phosphorylation is likely to have an important role in other polyglutamine disorders. However, the specific effects of phosphorylation may vary with disease and mutant protein. The Akt/PKB pathway has been implicated in HD (14). Insulin growth factor 1 (IGF-1) by its activation of Akt/PKB inhibits mutant huntingtin-induced neuronal cell death. Phosphorylation of huntingtin at serine 421 by Akt/PKB is required for the neuroprotective effects of IGF-1. The DRPLA protein (atrophin-1) is phosphorylated at serine 734 by c-Jun N-terminal kinase (JNK) in vitro (15). Kinetic studies found that mutant atrophin-1 had a modestly reduced affinity for JNK. This suggests that mutant atrophin-1 may be more slowly phosphorylated, thus delaying cellular processes critical for neuronal survival. The mutant form of the androgen receptor is toxic to cells, and caspase-mediated proteolysis of the androgen receptor enhances its toxicity. Recent experiments indicated that phosphorylation of the androgen receptor on serine 514 is required to enhance the ability of caspase-3 to cleave the androgen receptor and generate toxic fragments (16). The MEK1/2 MAP kinase pathway was shown to be necessary for this event. Interestingly, the expanded form of the androgen receptor was shown to have increased serine phosphorylation, which correlated with an increase in MAP kinase activation. Together, these studies point toward a role of cell signaling pathways in the pathogenesis of several polyglutamine disorders.

To further understand the biological role of phospho-S776 ataxin-1, we developed a cell culture assay for use in a screen for compounds that alter the phosphorylation of ataxin-1 S776. Here, we report the results of a screen of a library of 84 known kinase and phosphatase inhibitors.

RESULTS

Cell-culture based assay to measure ataxin-1 S776 phosphorylation

To develop a cell-culture based assay to monitor ataxin-1 S776 phosphorylation, we generated cell lines stably expressing an enhanced green fluorescent protein (EGFP) fused to various forms of ataxin-1. These included mutant ataxin-1 with 85 glutamine repeats or wild-type ataxin-1 with 30 glutamines, each with either a serine or an alanine at position 776 (Fig. 1A). Each of these lines was established from a single EGFP⁺ cell using fluorescence activated cell sorting (FACS). This resulted in cell lines in which >90% of the cells were EGFP⁺. In each of these four stably transfected cell lines, ataxin-1 was found predominantly in the nucleus (Fig. 1B).

To monitor phospho-S776 ataxin-1, we developed an enzyme-linked immunosorbent assay (ELISA). In the first step, a capture ELISA was performed in which EGFP–ataxin-1 was captured to the surface of a 96-well microplate with an anti-EGFP antibody. Secondly, a standard sandwich ELISA was performed using the phospho-S776 ataxin-1 specific antibody (PN1168) (11). The relative level of phosphorylation was determined by dividing the PN1168 ELISA readout by the EGFP fluorescence. For cells expressing EGFP–ataxin-1[30Q] or EGFP–ataxin-1[85Q], the amounts of EGFP fluorescence (Fig. 1C) and the PN1168 ELISA (Fig. 1D) were linear up to 50 µg of cell extract per well.

Experimental design and assay validation

Experiments were configured such that cells not expressing EGFP–ataxin-1 (CHO AA8) served as the negative control and cells expressing the EGFP–ataxin-1 in the absence of drug served as the positive control. The assay developed to screen the library is outlined in Figure 2A. Following the incubation of 30 000 cells per well for 24 h, cell lysates were prepared. From these, the EGFP–ataxin-1 protein was captured to the surface of a 96-well microplate. A standard sandwich ELISA using the phospho-S776 ataxin-1 specific antibody was performed. The relative amount of S776 phosphorylated ataxin-1 was determined by dividing the PN1168 signal by the EGFP fluorescence signal. To screen for modulators of ataxin-1 S776 phosphorylation, we screened the Screen-Well™ library consisting of 84 known kinase and phosphatase inhibitors. This commercially available library consists of 70 kinase inhibitors and 14 phosphatase inhibitors (Supplementary Material, Table S1). Cells expressing EGFP–ataxin-1 were screened using three concentrations of each drug (1, 10 and 25 µM).

A number of parameters were used to evaluate the effectiveness of our screening assay (17). The signal-to-noise ratio, defined as the mean signal subtracting the mean background signal divided by the standard deviation of the background signal, was 34.8 ± 6.1. A second parameter assessed was the signal-to-background ratio, which is the mean signal divided by the mean background. The signal-to-background ratio was 6.2 ± 1.1. Thus, both of these measured values were within the acceptable range for each parameter (17). The Z’-factor, a coefficient that is reflective of both the
assay signal dynamic range and the data variation associated with the control measurements (17), was also determined. Specifically, \( Z_0 \) assesses the assay itself without the intervention of test compounds. This factor represents the robustness of the assay after accounting for the dynamic range of the signal and variation associated with signal measurements. Typically, \( Z_0 \)-values greater than 0.2 are considered acceptable. The \( Z_0 \)-values in our experiments ranged from 0.3 to 0.6 (Fig. 2B). Finally, the variance of the relative phosphorylation measurements of the untreated cells was calculated. These values ranged from 5.0 to 16.5%.

Inhibitors of ataxin-1 phosphorylation

The results of the screen using cells expressing EGFP–ataxin-1[30Q] are shown in Figure 3A, and the results of the screen using cells expressing EGFP–ataxin-1[85Q] are shown in Figure 3B. The inhibitors are ordered from 1 through 84 (Supplementary Material, Table S1). The hit threshold or hit limit was designated as 2 SD from the mean of the control [dimethyl sulfoxide (DMSO) treated] ataxin-1 expressing cells. Setting the threshold at 2 SD provided a 95.4% confidence limit to the sample data. Setting the relative phosphorylation of control-treated cells at 1.0 resulted in a threshold twice the variance. Depending on the set of experiments, the threshold ranged 10–33%. Thus, at least a 10% change in S776 phosphorylation was detectable (Fig. 2B).

The frequency of hits varied depending on the concentration of inhibitor used. For cells expressing ataxin-1[30Q], 30, 11 and 10% of the compounds altered S776 phosphorylation at 25, 10 and 1 \( \mu \)M, respectively (Fig. 3C). For cells expressing ataxin-1[85Q], 30, 35 and 8% of the compounds altered S776 phosphorylation at 25, 10 and 1 \( \mu \)M, respectively (Fig. 3C). At all three concentrations tested, the vast majority of these hits (93/103, 90%) resulted in a decrease in the relative amount of ataxin-1 S776 phosphorylation. By measuring EGFP fluorescence, we were also able to assess whether the compounds had an effect on the cellular level of ataxin-1. The majority of the hits changed the protein level by \( \leq 15\% \). Of the hits with ataxin-1[85Q], 92% (56/61) changed the protein level by \( \leq 15\% \), while with ataxin-1[30Q] screen, 72% (30/42) changed the protein level by \( \leq 15\% \). The majority of these changes were reductions in EGFP–ataxin-1, suggesting some cytotoxicity at the concentration of the drugs used in this study. In contrast, staurosporine had a more dramatic effect on the levels of EGFP–ataxin-1. At a concentration of 1 \( \mu \)M, staurosporine reduced the amount of EGFP–ataxin-1[30Q] and EGFP–ataxin-1[85Q] by 30%. Staurosporine is a broad-spectrum inhibitor of protein kinases and it is known to be cytotoxic, even at low concentrations (18,19). Because of these reasons, we decided not to include this drug in future analyses.

Of interest, the compounds fell into three groups. The first group of compounds was those that had a similar effect on the phosphorylation of wild-type and mutant ataxin-1. In the case of cells expressing wild-type ataxin-1, this group included 56% of the hits at 25 \( \mu \)M, 100% of the hits at 10 \( \mu \)M and 38% of the hits at 1 \( \mu \)M. For cells expressing mutant ataxin-1, this group included 56% of the hits at 25 \( \mu \)M, 31% of the hits at 10 \( \mu \)M and 43% of the hits at 1 \( \mu \)M. The remaining two
groups consisted of compounds that affected the phosphorylation of only wild-type ataxin-1 or only mutant ataxin-1. For both wild-type and mutant ataxin-1, this was 44% of the hits at 25 μM, at 10 μM 0 and 69%, respectively, and at 1 μM 62 and 57%, respectively. Of note, there is some redundancy in the kinases/phosphatases inhibited by the compounds in the library. For further studies, we wanted to focus on those compounds whose effect was more likely to be physiologically relevant. To do this, we identified those drugs that affected ataxin-1 phosphorylation consistently in our experiments and at a concentration close to the IC50 for that drug. Using these criteria, and noting the redundancy in the library, we identified 17 compounds that modulated ataxin-1 S776 phosphorylation (Table 1).

Inhibitors of both wild-type and mutant ataxin-1 phosphorylation

Inhibitors of the epidermal growth factor and the platelet derived growth factor receptor tyrosine kinases (RTKs) inhibited the phosphorylation of ataxin-1[30Q] and ataxin-1[85Q] (Table 1). The RTK inhibitors tyrphostin-9 and -25 decreased phosphorylation of both wild-type and mutant ataxin-1 at the three concentrations tested. Once activated, many other signal-}

Inhibitors of only mutant ataxin-1 phosphorylation

The Src family of tyrosine kinases consists of nine members (24). Interestingly, a specific inhibitor (damnacanthal) of one of the members of this family (p56, Lck) preferentially decreased the phosphorylation of mutant ataxin-1 (Table 1). Another family of kinases that are known to be involved in RTK signaling are the tyrosine kinases known as the Janus activated kinases (JAKs) (25). We found that an inhibitor of JAK2 and JAK3 (AG490) preferentially decreased the relative phosphorylation of mutant ataxin-1. Together, these data indicate that signaling pathways involving calcium signaling are involved in ataxin-1 phosphorylation.
Figure 3. The results of the screen of the 84 known kinase and phosphatase inhibitors. The 84 inhibitors are depicted on the horizontal axis (1 through 84, Supplementary Material, Table S1). The relative phosphorylation of untreated cells was set at 1.0 to which the treated samples were normalized. For a drug to be designated a hit, the average change in relative phosphorylation had to be \( \geq 2 \) SD from the average relative phosphorylation of the DMSO-treated samples for all of the experiments at the indicated concentration. (A) Results of screening cells that express EGFP–ataxin-1[30Q]. (B) Results of screening cells that express EGFP–ataxin-1[85Q]. In both sets of data gray bars indicate no change in the relative level of phosphorylation. Yellow bars represent drugs that only modulated wild-type ataxin-1 phosphorylation at the indicated concentration. Red bars represent drugs that only modulated mutant ataxin-1 phosphorylation at the indicated concentration. Blue bars represent those drugs that modulated both wild-type and mutant ataxin-1 phosphorylation at the specified concentration. (C) Summary of the number and frequency of hits at each of the concentrations screened for cells expressing either wild-type or mutant ataxin-1.
leads to the generation of phosphatidylinositol-3,4,5-triphosphate, which recruits inactive Akt/PKB from the cytosol to the plasma membrane via a pleckstrin homology domain. This results in the phosphorylation of Akt/PKB on T308 and S473, which fully activates the kinase to phosphorylate its substrates. We found that two inhibitors of PI3K, quercetin dihydrate and wortmannin, preferentially decreased phosphorylation of mutant ataxin-1. Numerous reports have implicated the Syk tyrosine kinase in the activation of PI3K (26,27). Similar to the findings using the PI3K inhibitors, the Syk inhibitor piceatannol preferentially decreased phosphorylation of mutant ataxin-1 at 10 μM. At the higher concentration of 25 μM, piceatannol had effects on both wild-type and mutant ataxin-1. The myosin light chain kinase inhibitor ML-9 has also proven to be useful inhibitor of Akt/PKB when used at concentrations between 10 and 50 μM (28). ML-9 selectively diminished phosphorylation of ataxin-[85Q]. Moreover, this inhibition was only observed at inhibitor concentrations of 10 and 25 μM, within the range that inhibits Akt/PKB.

### Activation of the PI3K/Akt pathway by ataxin-[85Q]

To investigate whether the specific effect of PI3K/Akt inhibitors on the phosphorylation of ataxin-[85Q] might reflect the activation of the PI3K/Akt pathway by ataxin-[85Q], we examined the relationship between ataxin-1 expression (wild-type or mutant) and the level of activated Akt/PKB in both tissue culture cells and SCA1 transgenic mice. Using an Akt/PKB kinase assay, in tissue culture cells expressing mutant ataxin-[85Q], there was a 4-fold increase in the amount of active Akt/PKB activity when compared with cells expressing wild-type ataxin-[30Q] (Fig. 4A). To assess Akt/PKB activity in vivo, the phosphorylation status of Akt/PKB at serine 473 was assessed in cerebellar extracts prepared from SCA1 transgenic mice (29), using an ELISA assay. A 3-fold increase in the amount of active Akt/PKB was detected in cerebellar extracts from transgenic mice expressing mutant ataxin-[82Q], B05 mice, when compared with the amount of active Akt/PKB seen in extracts from mice expressing wild-type ataxin-[30Q], A02 mice, (Fig. 4B). The results from the ELISA experiments for active Akt/PKB were confirmed by western blot analysis using an antibody that recognized total Akt/PKB and an antibody that recognized only S473 phosphorylated Akt/PKB (Fig. 4C). Similar to the ELISA-based assay, a 2-fold increase in the amount of active Akt/PKB was detected in cerebellar extracts from the B05 mice when compared with the amount of active Akt/PKB seen in cerebellar extracts from the A02 mice. The amount of active Akt/PKB in the A02 mice expressing ataxin-[30Q] was 50% higher than that observed in wild-type mice expressing murine endogenous ataxin-1 with two glutamine repeats.

### DISCUSSION

In this report, a cell-culture based assay to screen for modulators of ataxin-1 S776 phosphorylation is described. In an effort to elucidate the cell signaling pathway(s) leading to ataxin-1 S776 phosphorylation, this assay was used to screen a library of known kinase and phosphatase inhibitors. The results of this screen revealed that two pathways can affect the phosphorylation of ataxin-1 at S776 (Fig. 5). One pathway, perhaps involving calcium signaling, likely involves the normal physiological role of S776 phosphorylated ataxin-1. The second pathway specifically affects the phosphorylation of mutant ataxin-1 and appears to result from the activation of the Akt/PKB signaling pathway by mutant ataxin-1.

The assay developed to monitor the phosphorylation of ataxin-1 at serine 776 is very robust. The background was low, i.e. the output from cells expressing ataxin-1 with an

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### Table 1. Summary of compounds that modulate ataxin-1 S776 phosphorylation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Kinase or phosphatase</th>
<th>Ataxin-1-[30Q] (% inhibition)</th>
<th>Ataxin-1-[85Q] (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantharidic acid</td>
<td>25</td>
<td>PP1/PP2A</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>Cantharinin</td>
<td>25</td>
<td>PP1</td>
<td>40</td>
<td>-11</td>
</tr>
<tr>
<td>Tyrphostin-9</td>
<td>10</td>
<td>PDGFRe</td>
<td>40</td>
<td>57</td>
</tr>
<tr>
<td>Tyrphostin-25</td>
<td>25</td>
<td>EGFR</td>
<td>29</td>
<td>28</td>
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<tr>
<td>PPI</td>
<td>25</td>
<td>SRC family</td>
<td>63</td>
<td>74</td>
</tr>
<tr>
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<td>25</td>
<td>PKC</td>
<td>42</td>
<td>53</td>
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<td>KN-95</td>
<td>25</td>
<td>CamKII</td>
<td>22</td>
<td>61</td>
</tr>
<tr>
<td>Ro 31-8220</td>
<td>25</td>
<td>PKC/CamKII</td>
<td>53</td>
<td>63</td>
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<td>5-Iodotubercidin</td>
<td>10</td>
<td>ERK2</td>
<td>56</td>
<td>77</td>
</tr>
<tr>
<td>Damnacanthal</td>
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<td>p56</td>
<td>5</td>
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<tr>
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<td>25</td>
<td>JAK</td>
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<tr>
<td>H-89</td>
<td>10</td>
<td>PKA</td>
<td>-1</td>
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<td>10</td>
<td>Cdk</td>
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<tr>
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<td>PI3K</td>
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<tr>
<td>Wortmannin</td>
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<td>ML-9</td>
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<td>PKB/Akt</td>
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<tr>
<td>Piceatannol</td>
<td>10</td>
<td>Syk</td>
<td>8</td>
<td>36</td>
</tr>
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</table>

*Compounds are grouped by their effect on phosphorylation. Bold rows: modulates wild-type ataxin-1; roman rows: modulates both wild-type and mutant ataxin-1; italic rows: modulates only mutant ataxin-1.

*Data on the inhibition of ataxin-1 S776 phosphorylation is shown for each compound at or above the IC₅₀ value for each inhibitor.

*A negative value for the percent inhibition indicates an increase in the relative phosphorylation.
Alanine at position 776 was the same as that of cells not expressing ataxin-1. In addition, the assay was very reproducible. The variance among untreated samples was between 5 and 17%, thus it was possible to readily detect changes in the relative level of phosphorylation as small as 10%. The assay, as designed, has the potential to be fully automated because it uses a 96-well plate format and two standard readouts, EGFP fluorescence and optical density at 450 nm. In the kinase/phosphatase inhibitor screen, three distinct classes of inhibitors of ataxin-1 phosphorylation were found: those that modulated the phosphorylation of both wild-type and mutant ataxin-1, those that only altered the phosphorylation of mutant ataxin-1 and those that affected the phosphorylation of only wild-type ataxin-1. We suggest that those inhibitors that affected the phosphorylation status of both wild-type and mutant ataxin-1 were directed at the normal cell signaling pathways involved in the phosphorylation of S776 in ataxin-1.

The group of inhibitors that modulated both wild-type and mutant ataxin-1 included those that inhibited growth factor RTKs, PKC, CaMKII, ERK2 and the Src family of tyrosine kinases. At this time, the only evidence that any of these kinases might be directly involved in the phosphorylation of ataxin-1 is for CaMKII, where its consensus recognition sequence matches the sequence of ataxin-1 around S776. Of interest, all of these kinases can be associated with intracellular calcium signaling. Src, likely with phospholipase C, is involved in the intracellular release of calcium stores from the endoplasmic reticulum (30,31). The release of these stores is involved in the activation of PKC, CaMKII and ERK2 (22,23,32,33).

In contrast, it is suggested that the inhibitors that altered only the phosphorylation of mutant ataxin-1 likely target pathways that are activated in response to the presence of mutant ataxin-1. This group included inhibitors of the PI3K/Akt pathway preferentially phosphorylates mutant ataxin-1 and thus may be involved in SCA1 pathogenesis. Elements in this latter pathway include the components and regulators of PI3K/Akt cell signaling.

Figure 4. Mutant ataxin-1 is associated with increased Akt/PKB activity. (A) In vitro kinase assay using cell culture extracts from cells expressing wild-type ataxin-1[30Q] (gray bar) or mutant ataxin-1[85Q] (black bar). The readout of the assay was phosphorylation of a GSK3 peptide, an endogenous substrate of Akt/PKB. The amount of phosphorylated GSK3 peptide was normalized to the total amount of Akt/PKB in the lysate. (B) An ELISA assay was used to determine the total amount of Akt/PKB and the amount of S473 phosphorylated Akt/PKB in cerebellar extracts from mice expressing either ataxin-1[30Q] (A02 mice) (gray bar) or from mice expressing ataxin-1[82Q] (B05 mice) (black bar). The amount of active Akt/PKB (S473 phosphorylated) was normalized to the total amount of Akt/PKB in the extract. In both (A) and (B), ataxin-1[30Q] expressing cells and mice had the same level of AKT as seen in untransfected cells or non-transgenic mice. (C) Western blot analyses of cerebellar extracts from B05 mice expressing mutant ataxin-1[82Q], A02 mice expressing ataxin-1[30Q] and wild-type mice probed with either a total Akt/PKB antibody or one specific for phospho-S473 Akt/PKB. The amount of active Akt/PKB was measured in the cerebellar extracts by densitometric analysis followed by normalization to the total amount of Akt/PKB in the extract. The quantitative analysis is an average of three determinations.
pathway, JAK tyrosine kinases, cyclin-dependent kinase (CDK), PKA, p56 and the spleen tyrosine kinase (Syk). JAKs are involved in the activation of PI3K, a kinase upstream of Akt/PKB (34). Syk has been reported to be involved in the regulation of PI3K (26,27). Thus, these data support the previous finding implicating Akt/PKB in SCA1 pathogenesis (12). The role that (CDKs) or PKA have in ataxin-1 phosphorylation is unknown. However, both can be linked to the PI3K/Akt pathway. CDK5 is involved in neuronal survival pathways through its activation of PI3K and Akt/PKB (35). Akt/PKB can be activated by PKA through a PI3K-independent pathway (36).

Both in tissue culture cells and in cerebella expressing mutant ataxin-1, there was an increased level of active Akt/PKB. This suggests that one consequence of polyglutamine tract expansion is the misregulation of cell signaling pathways involving Akt/PKB. The mechanism by which an expanded polyglutamine tract activates the Akt/PKB pathway is yet to be determined. Our data suggest two kinases upstream of Akt/PKB as possible points for regulation of the mutant ataxin-1-specific pathway. First, the JAK inhibitor AG-490 preferentially inhibited mutant ataxin-1 phosphorylation. Secondly, the p56 inhibitor damanacanthal preferentially diminishes mutant ataxin-1 phosphorylation. Mutant ataxin-1-associated activation of either JAKs or p56 (or both) is predicted to activate the PI3K pathway, resulting in increased levels of active Akt/PKB, and thus more phosphorylated mutant ataxin-1. One hypothesis is that these two kinases are part of a ‘molecular switch’, redirecting mutant ataxin-1 into the PI3K/Akt pathway. The trigger for this switch, whether it is the expanded repeat itself or a toxic property that results from mutant ataxin-1 expression, remains to be determined.

The concept that expression of mutant ataxin-1 results in the misregulation of cell signaling pathways is supported by previous studies. For example, a link between an alteration in calcium homeostasis and SCA1 pathogenesis was previously reported (37). Interestingly, an intracellular calcium channel (IP3R1) and the calcium pump (SERCA2) were downregulated in transgenic mice expressing mutant ataxin-1. IP3R1 increases cytoplasmic calcium, whereas SERCA2 decreases cytoplasmic calcium by pumping it into the endoplasmic reticulum. The net effect on cytoplasmic calcium stores through downregulation of IP3R1 and SERCA2 by mutant ataxin-1 depends on the relative contribution of IP3R1 and SERCA2 in controlling calcium levels. Nonetheless, downregulation of these genes may result in a perturbation in calcium homeostasis, which subsequently could modulate the calcium-associated pathway responsible for phosphorylation of both wild-type and mutant ataxin-1. A cellular consequence of this may be a preferential phosphorylation of mutant ataxin-1 by diverting the protein into the mutant ataxin-1-specific pathway (i.e. the PI3K/Akt pathway, Fig. 5). The misregulation of cell signaling pathways has been implicated in other polyglutamine disorders, most notably HD. The IGF-1 signaling pathway, signaling through Akt/PKB, has been shown to be a key modifier of huntingtin pathogenesis (14). In support of this pathway, the level of Akt/PKB is altered in HD brains. In the striatum, a prime site of pathology in HD, the level of full-length Akt/PKB was dramatically reduced. In a cell-culture model, expression of a constitutively active form of Akt/PKB resulted in fewer inclusions and increased cell survival. Another kinase in the IGF-1 signaling pathway, SGK (serum- and glucocorticoid-induced kinase), has also been recently shown to phosphorylate huntingtin on serine 421 (38). Activation of SGK by mutant huntingtin is mediated through the p38/MAPK pathway. Unlike Akt/PKB, the level of SGK is increased in HD brains, supporting the findings that SGK participates in a neuronal survival pathway, which is mediated by IGF-1. In contrast to the findings in SCA1, phosphorylation of huntingtin results in a reduction in intranuclear inclusions and an increase in cell survival. Thus, suppression of the PI3K/Akt cell-signaling pathway may be involved in HD pathogenesis.

The results presented here along with those reported previously suggest that alterations in signal transduction pathways have a role in expanded polyglutamine mediated pathogenesis. Furthermore, the results of this study indicate that mutant ataxin-1 can be phosphorylated by a pathway activated in response to its expression and that this pathway does not regulate the phosphorylation of wild-type ataxin-1. This suggests that it might be possible to identify drugs whose effects are restricted to mutant ataxin-1.

MATERIALS AND METHODS

Plasmids, cell culture and transfection

To generate an EGFP–SCA1 fusion, a SCA1 human cDNA contained on an EcoR1–SalI fragment was subcloned into the EcoRI and SalI sites of pEGFP-C2 (BD Biosciences). Two plasmids were generated, one with 30 CAG repeats and the other with 85 CAG repeats. The Nhel–SalI fragment from this plasmid, containing the EGFP–SCA1 fusion, was subcloned into the Nhel–SalI sites of pTRE2 (BD Biosciences). To incorporate the serine into alanine mutation at amino acid 776, the BstEII–NarI fragment from the EGFP–SCA1 plasmid (containing either 30 or 85 polyglutamines) was replaced with the BsrEI–NarI fragment from pCDNA-FLAG ataxin-1[30Q]-A776 (11). The length of the polyglutamine tract and the amino acid at position 776 was confirmed by DNA sequence analysis.

Cho-AA8 Tet-off cells (BD Biosciences) were cultured in MEMα media supplemented with FBS (10%), penicillin and streptomycin (50 U/ml) and genetin (100 μg/ml). To generate stable cell lines expressing EGFP–ataxin-1, Cho-AA8 cells were co-transfected with the EGFP–SCA1 plasmid and the plasmid containing the hygromycin B selectable marker (pTK-Hyg, BD Biosciences). The cells were transfected with Lipofectamine and the Plus reagent as per the manufacturer’s recommended protocol (Invitrogen). Hygromycin-resistant cells were selected by growing the transfected cells in media supplemented with 400 μg/ml hygromycin B (Invitrogen). The media was changed every third day for 2 weeks, at which time hygromycin-resistant clones were picked. The clones were expanded either in the presence (1 μg/ml) or in the absence of doxycycline. The cells grown in the absence of doxycycline were analyzed by fluorescent microscopy to assess the subcellular localization of the EGFP–ataxin-1 fusion protein. Protein lysates were prepared and analyzed...
by western blot to ensure that the proper size fusion protein was generated (11).

Subcloning of cell lines by FACS
When the clones were analyzed by fluorescent microscopy, we found a low percentage of EGFP+ cells (10–40%). To increase the percentage of cells expressing EGFP–ataxin-1, we subcloned each of the lines by FACS. This was performed at the flow cytometry core facility in the Cancer Center at the University of Minnesota. A single EGFP+ cell was placed into a well of a 96-well tissue culture plate. The cells were grown and analyzed as described earlier.

Kinase and phosphatase inhibitor treatment
Cells expressing either EGFP–ataxin-1[30Q] or EGFP–ataxin-1[85Q] were plated onto 96-well plates (30 000 cells per well). The Screen-WellTM library of 84 known kinase and phosphatase inhibitors (Biomol) is supplied in a 96-well plate as 10 mM stock solutions. Prior to adding the inhibitors to the cells, the 10 mM stock solutions were diluted in DMSO and then in tissue culture media. The final concentration of DMSO in the cell culture media was ≤0.5% (vol/vol). After the addition of the inhibitor library, the cells were incubated for 24 h at 37°C in 5% CO2.

Enzyme-linked immunosorbent assay
After treatment, lysates were prepared by washing the plate two times with phosphate buffered saline (PBS) followed by the addition of 150 μl of lysis buffer [50 mM Tris–Cl pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, 1× protease inhibitor (Roche Biochemicals), 1× phosphatase inhibitor cocktails 1 and 2 (Sigma)]. The plate was rocked at 4°C for 15 min followed by centrifugation at 1000g at 4°C for 10 min. The cleared lysate (135 μl) was transferred to a new 96-well plate and directly used to run the assay or stored at −20°C. To run the assay, a black 96-well plate precoated with a goat anti-EGFP antibody (Pierce Biotechnology) was washed three times with PBST (1×PBS with 0.05% Tween-20). The cleared lysate (100 μl) was added to the washed black plate and rocked at room temperature for 60 min. The plate was subsequently washed three times with PBST (1×PBS with 0.05% Tween-20) followed by the addition of 125 μl of PBST. The EGFP fluorescence was read on a fluorescent microplate reader (Bio-Tek Instruments). The PBST was aspirated from the plate, and the primary antibody (1:1000, PN1168) (11) in 1×PBS (100 μl) was added to the plate and rocked at room temperature for 60 min. The plate was washed as described earlier and the secondary antibody (1:2000, goat anti-Rabbit HRP, Jackson Immunoresearch) in 1×PBS containing 1 mg/ml bovine serum albumin and 0.1% Tween-20 was added to each well (100 μl per well) followed by rocking at room temperature for 60 min. The plate was washed as described earlier and developed using 100 μl of TMB (slow kinetic form, Sigma). To stop the reaction, 50 μl of 4 N H2SO4 was added. To quantify the assay, the reaction was transferred to a clear 96-well plate and the absorbance of the yellow product was read at 450 nm.

Data analysis
Data from the ataxin-1 S776 phosphorylation assay was processed and analyzed as follows. For both the EGFP capture ELISA and the PN1168 sandwich ELISA, cells not expressing EGFP–ataxin-1 (CHO AA8) were used as a negative control. For each ELISA, the raw data were first scaled to the negative control (arbitrarily set to 1.0), by multiplying each of the values by a scaling factor. For our analyses, the scaling factor was defined as the inverse of the average readout of the negative control for each of the ELISA assays. To determine the relative level of ataxin-1 S776 phosphorylation, we divided the scaled PN1168 ELISA result by the scaled EGFP capture ELISA result. This result was then scaled to the relative phosphorylation of the untreated cells (arbitrarily set to 1.0). We chose a hit threshold of 2 SD away from the relative phosphorylation of the untreated cells.

Determination of the relative amount of active Akt/PKB
To compare the amount of active (phosphorylated) Akt/PKB in tissue culture cells expressing either wild-type or mutant ataxin-1, we performed an in vitro Akt/PKB kinase assay. The assay was performed as per the manufacturers recommended protocol (Cell Signaling Technology). Briefly, immobilized Akt/PKB monoclonal antibody was used to capture, via immunoprecipitation, Akt/PKB from cell extracts prepared from cells expressing either wild-type or mutant ataxin-1. A synthetic GSK-3 fusion protein (an Akt/PKB substrate) was added to the immunoprecipitated Akt/PKB. The amount of phosphorylated GSK-3 fusion protein was determined by western blot analysis with a phospho-GSK-3 specific antibody. The amount of phospho-GSK-3 was normalized to the total amount of immunoprecipitated Akt/PKB. The amount of phosphorylated GSK-3 fusion protein was determined by western blot analysis with a phospho-GSK-3 specific antibody. The amount of phosphorylated GSK-3 was normalized to the total amount of immunoprecipitated Akt/PKB (determined by probing the western blot with an Akt/PKB antibody).

We used two separate ELISAs to determine the amount of active Akt/PKB in cerebellar extracts from transgenic mice expressing either wild-type ataxin-1 (A02) or mutant ataxin-1 (B05) (29). To determine the total amount of Akt/PKB (both S473 phosphorylated and unphosphorylated) present in the cerebellar extracts, we performed a total Akt/PKB ELISA (Biosource) using 100 μg of extract. Cerebellar extracts were prepared according to the manufacturer’s recommended protocol. To measure the amount of active Akt/PKB (S473 phosphorylated) in the cerebellar extract, we used an Akt/PKB ELISA (Biosource) using 100 μg of total cerebellar extract for this experiment. The relative amount of active Akt/PKB was determined by normalizing the S473 phosphorylated Akt/PKB to the total amount of Akt/PKB in the cerebellar lysate.

Western blot analysis
Cerebellar extracts were prepared by homogenizing one-half of a cerebellum in 0.25 M Tris–Cl pH 7.5 containing 1× protease inhibitors (Roche Biochemicals) and phosphatase inhibitor cocktails 1 and 2 (Sigma). Lysates were prepared by three rounds of freezing the homogenate in liquid nitrogen followed by thawing at 37°C. The lysate was cleared by centrifugation at 750g for 10 min at 4°C. The protein concentration was
REFERENCES


