Identification of a novel phosphorylation site in ataxin-1

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Abstract


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1. Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that belongs to a family of neurodegenerative disorders including Huntington’s disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), and several of the spinocerebellar ataxias 2,3,6,7 and 17 [3,4]. These disorders are caused by an expansion of CAG repeats encoding a polyglutamine tract. Although the length of the polyglutamine tract determines the age of onset and severity in many of these diseases [5], it has been suggested that amino acids outside of the polyglutamine tract may control the course of disease [6].

Phosphorylation of ataxin-1 at S776 further shows the importance of phosphorylation in polyglutamine repeat disorders [1,2]. It was shown that protein–protein interaction and/or phosphorylation plays an important role in many neurodegenerative diseases such as SBMA, SCA1, AD, and HD [1,2,7–9]. Phosphorylation of huntingtin via the IGF-1/ Akt pathway was found to be neuroprotective and is able to inhibit neuronal death [7]. Therefore, in this communication, phosphorylation of ataxin-1 on residues other than serine 776 is investigated and demonstrated. A novel
phosphorylation site within ataxin-1 is described. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed, which produced spectra of an ataxin-1 peptide containing one phosphate. Tandem mass spectrometry (MS/MS) was performed indicating the peptide to be singly phosphorylated on threonine 236 or serine 239. Finally, mutations of threonine 236 and serine 239 to alanine along with MALDI-TOF MS and MS/MS confirmed phosphorylation at site serine 239.

2. Materials and methods

2.1. Cloning

Transient transfections were done using the pCDNA1 amp eukaryotic expression vector containing the FLAG-tagged ataxin-1 [30Q] cDNA [10]. Threonine 236 and serine 239 were mutated using the Quikchange site-directed mutagenesis kit (Stratagene). The codons ACC and TCC producing threonine 236 and serine 239 were mutated to GCC and GCA, respectively, to produce ataxin-1 [30Q] T236A and ataxin-1 [30Q] S239A.

2.2. Immunoprecipititation, purification and digestion of ataxin-1

COS1 cells were transiently transfected with 2 μg of Flag tagged ataxin-1 [30Q] cDNA using Lipofectamine™ (Invitrogen). Forty-eight hours post transfection, the cells were washed two times in cold 1× PBS and lysed in 500 μl of buffer (50 mM Tris–HCl [pH 7.5], 2.5 mM MgCl2, 100 mM NaCl, 0.5% Triton-X, 1× protease inhibitors [Roche Biochemicals], phosphatase inhibitor cocktails 1 and II [Sigma]). The DNA was sheared through a 21-gauge syringe 10 times followed by a 25-gauge syringe five times. Clearing of the lysate was performed at 4 °C by centrifugation at 14,000 rpm for 10 min. Immuno-precipitation was done by adding the supernatant to 1 μl of monoclonal VP16 IgG1 and 50 μl of 50% protein-G-sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. The preclearing mixture was then centrifuged at 14,000 rpm for 3 min. Following preclearing, the supernatant was then immunoprecipitated with 1 μl of anti-Flag (Sigma) and 50 μl of 50% protein-G-sepharose beads (Amersham Pharmacia Biotech) by rotating overnight at 4 °C. Immuno-complexes were then collected by centrifugation at 14,000 rpm for 1 min and washed three times in lysis buffer. The samples were then boiled for 5 min in 6× SDS sample loading buffer and fractionated on a 4–12% Bis-Tris acrylamide gel (Invitrogen). Visualization of ataxin-1 was done using SilverQuest™ silver staining kit following the Fast Staining Protocol included. Silver destaining was done by cutting out the band of interest and incubating in 300 μl of a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 8 min, followed by four 8-min washes in distilled water. Finally, the band is incubated in 100% acetonitrile, and then dried using a speed-vacuum. Endoprotease digestion was done on the dried destained bands. The bands were incubated at 37 °C for trypsin and 30 °C for chymotrypsin overnight in 50 μl of a solution containing 12.5 ng/μl of trypsin or chymotrypsin in 100 mM Tris–HCl containing 10 mM CaCl2, pH7.8. Following incubation, the supernatant was removed and retained. Thirty microliters of 20 mM ammonium bicarbonate was then added to the bands and incubated for 20 min. The supernatant was pooled with that of the previous step. The bands were then washed three times with 30 μl of 50% acetonitrile, 5% formic acid with the supernatants being pooled as before. The supernatant containing the peptides was then dried using a speed-vacuum. The samples were then stored at −80 °C.

2.3. MALDI-TOF MS

Samples were prepared for MALDI-TOF MS with a Millipore ZipTipC18 according to the manufacturer’s protocol. To crystallize the samples, 1 μl of 20 mg/ml alpha-cyano-4-hydroxy-trans-cinnamic acid (CCA) in 0.1% TFA/50% acetonitrile (Sigma-Aldrich, St. Louis, MO) was added to the eluted peptides. The peptides were then spotted onto the MALDI target and dried. Full scans of the peptide mixture from 500–3500 m/z and tandem mass spectra of select ions were collected on a QSTAR XL (Applied Biosystems) quadrupole time-of-flight mass spectrometer with an orthogonal MALDI source. Mass accuracy was calculated in parts per million (ppm) by dividing the difference between the experimental and theoretical m/z values by the theoretical m/z value then multiplying by 106. MS/MS was performed by gating on the peptide of interest, from immunoprecipitated material isolated from transfected cells and used in the full scan. All peaks labeled were three times above background. b and y ions are labeled above the corresponding peak in the spectra. Degree signs denote the loss of a water and asterisks denote loss of NH3 from a fragment ion [14]. Ionmonium ions are labeled with an I and int indicates internal fragmentation. The data were collected, smoothed and centroided in BioAnalyst (ABI), and then plotted in GraphPad Prism using m/z to percent intensity values. (A) Full scan of ataxin-1 masses are monoisotopic [MH]+. (*) Peptide 229–249 without modifications. (**) Peptide 229–248 with one phosphate. (B) MS/MS ion spectrum of the peptide SRAPGLITPG(phos-S)PAPQQNY formed by MALDI ionization. The amino acid sequence is displayed above the spectrum (phosphorylated residue in larger font) and corresponds to residues 229–248 of ataxin-1. (C) MS/MS ion spectrum of the peptide SRAPGLITPG(phos-S)PPAPQQNY formed by MALDI ionization. The amino acid sequence is displayed above the spectrum (phosphorylated residue in larger font) and corresponds to residues 229–248 of ataxin-1.
mass spectral data of select ions were collected on a QSTAR XL (Applied Biosystems Inc., Foster City, CA) quadrupole time-of-flight mass spectrometer with an orthogonal MALDI source. The TOF region acceleration voltage was 4 kV and the injection pulse repetition rate was 6.0 kHz. Laser pulses were generated with a nitrogen laser at 337 nm, ~9 μJ of laser energy using a laser repetition rate of 20 Hz. Mass spectra were the average of approximately 50–200 laser shots collected in positive mode. External calibration was performed using human angiotensin II (monoisotopic [MH+] m/z 1046.5417; Sigma) and adrenocorticotropic hormone (ACTH) fragment 18–39 (monoisotopic [MH+] m/z 2465.1989; Sigma). Mass accuracy was calculated in parts per million (ppm) by dividing the difference between the experimental and theoretical m/z values by the theoretical m/z value then multiplying by 10^6. MS/MS was performed by gating on the peptide of interest.

3. Results

3.1. MALDI-TOF MS analysis of ataxin-1 chymotryptic digest yields phosphorylated and non-phosphorylated form of a single peptide

Initial investigations of the phosphorylation state of ataxin-1 were done by tryptic digestion of ataxin-1 followed by HPLC and mass spectrometry. This method revealed only one site of phosphorylation with only 30% coverage of the protein [1]. The limited coverage could be explained by the use of trypsin as the endoprotease, which generates large peptides that are outside the detection of the mass spectrometer. Therefore, to increase the coverage, chymotrypsin, which cleaves ataxin-1 into smaller peptides, was chosen to digest ataxin-1 prior to MALDI-TOF MS. This digest produced peptides not seen previously, such as both the phosphorylated and unphosphorylated forms of peptide 229–248. Fig. 1A shows a representative MALDI-TOF mass spectrum of peptides generated from chymotrypsin digestion of immunopurified ataxin-1 from transiently transfected COS1 cells. Inspection of the spectrum indicated a peak at 2159.0324 m/z that matched a theoretical m/z of 2159.0287 corresponding to the ataxin-1 peptide 229–248 plus one phosphate. An additional peak at 2079.0600 m/z matched the theoretical m/z ratio 2079.0623 m/z of peptide 229–248 with no modifications. These results suggest peptide 229–248 contains a putative phosphorylation site.

3.2. MS/MS fragmentation of peptide 2159.0287 m/z from ataxin-1 yields sequence corresponding to the peptide 229–248 containing one phosphate

MS/MS was performed to confirm the amino acid sequence and identify the potential site of phosphorylation. The peptide found at 2159.0324 m/z represents the residues 229–248 of ataxin-1 plus one phosphate. Within

Table 1

<table>
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<th>Residues</th>
<th>Sequence</th>
<th>Ion</th>
<th>Experimental m/z</th>
<th>Theoretical m/z</th>
<th>ppm</th>
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</thead>
<tbody>
<tr>
<td>229–237</td>
<td>SRAPGLI(PHOS-T)P</td>
<td>b9</td>
<td>974.4948</td>
<td>974.49</td>
<td>4.93</td>
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<tr>
<td>229–238</td>
<td>SRAPGLI(PHOS-T)PG</td>
<td>b10</td>
<td>1030.518</td>
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<td>7.76</td>
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<tr>
<td>235–248</td>
<td>PGSPPPAAQNQY</td>
<td>y10</td>
<td>1129.541</td>
<td>1129.53</td>
<td>9.74</td>
</tr>
<tr>
<td>237–248</td>
<td>SPPPAAQNQY</td>
<td>y12</td>
<td>1283.617</td>
<td>1283.60</td>
<td>13.24</td>
</tr>
</tbody>
</table>

(A) Sequence for peptide 229–248 with the corresponding b and y ions matched to the fragmentation pattern for phospho-threonine at position 236. The red box surrounds the ions critical for determination of phospho-threonine at position 236. Theoretical and experimental masses are the monoisotopic [MH+] masses for the indicated ions, b9, b10, y10 and y12, followed by the calculated ppm. (B) Sequence for peptide 229–248 with the corresponding b and y ions matched to the fragmentation pattern for phospho-serine at position 239. The red box surrounds the ions critical for determination of phospho-serine at position 239. Theoretical and experimental masses are the monoisotopic masses for the indicated ions, b8, b9, b10 and y11, followed by the calculated ppm.
this peptide there are two possible sites of phosphorylation: at threonine 236 and serine 239. To distinguish between the two possible sites, the peptide fragmentation was analyzed for phosphorylation of either threonine 236 or serine 239. In either case, 7 of 17 y ions and 9 of 17 b ions were identified, along with several internal fragments, ammonium ions and neutral loss of water and ammonium. The large abundance of internal fragments results from the presence of five prolines in this peptide (Fig. 1B and C).

The presence of two ions (b11-H3PO4 and y20-H3PO4) lacking a phosphate group was also observed. These results confirm the identity of peptide 2159.0324 m/z as residues 229-SRAPGLITPGSPPPAQQNQY-248 containing one phosphate group. Distinguishing between threonine 236 and serine 239 as the site of phosphorylation was difficult due to the close proximity of the two residues, which limited the number of diagnostic b and y ions that could be used. When considering threonine 236 as the

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**Fig. 2.** MS/MS of ataxin-1 with threonine 236 mutated to alanine. Samples were prepared for MALDI-TOF MS/MS with a Millipore ZipTipC18 according to the manufacturer’s protocol. Full scans of ataxin-1 T236A digested with chymotrypsin were collected from 500–3500 m/z on a MALDI QSTAR XL quadrupole time-of-flight mass spectrometer. MS/MS was performed by gating on the peptide of interest, from immunoprecipitated material isolated from transfected cells and used in the full scan. All peaks labeled were three times above background. b and y ions are labeled above the corresponding peak in the spectrums. Degree signs denote the loss of a water and asterisks denote loss of NH3 from a fragment ion [14]. Immonium ions are labeled with an I and int indicates internal fragmentation. The data were plotted in GraphPad Prism using m/z values that were smoothed and centroided in BioAnalyst (ABI). (A) Expanded view of peak at 2049.0518 m/z formed by MALDI ionization and the MS/MS ion spectrum for that peptide representing the peak. The amino acid sequence is displayed above the spectrum and corresponds to residues 229–248 of ataxin-1 T236A. (B) Expanded view of peak at 2129.0181 m/z formed by MALDI ionization and the MS/MS ion spectrum for that peptide representing the peak. The amino acid sequence is displayed above the spectrum (phosphorylated residue in larger font) and corresponds to residues 229-(phos-S239)-248 of ataxin-1 T236A.
putative phosphorylation site (Fig. 1B, Table 1A), the presence of y10 and y12 with no phosphate is consistent with an unphosphorylated serine 239. The $m/z$ for ions b9 and b10 was consistent with the addition of a phosphate. These results support phosphorylation of threonine 236. When considering serine 239 as the putative phosphorylation site (Fig. 1C, Table 1B), the presence of ions b8, b9 and b10 without a phosphate is consistent with unphosphorylated threonine 236. The $m/z$ for ion y11 was consistent with the addition of a phosphate and therefore supports phosphorylation of serine 239. Thus, these results unambiguously show that the peak at 2159.0324 $m/z$ is peptide 229–248 containing one phosphate group. Further, the fragmentation pattern suggests the peptide consists of a mixture of two mono-phosphorylated species, one with phospho-serine and the other with phospho-threonine. However, because of the proximity of the two residues, further analyses were performed.

3.3. MALDI-TOF MS of mutations confirms phosphorylation at serine 239 but fails to confirm phosphorylation at threonine 236

To examine the phosphorylation status of threonine 236 and serine 239, each site was mutated to an alanine using the Quickchange site-directed mutagenesis kit. The codons ACC and TCC producing threonine 236 and serine 239 were mutated to GCC and GCA, respectively, to produce ataxin-1 [30Q] T236A and ataxin-1 [30Q] S239A. Sequencing of the plasmid DNA confirmed both the mutations and the CAG repeat length. The effect of single residue mutations was then analyzed by MALDI-TOF MS. In-gel chymotryptic digest of immuno-purified ataxin-1 [30Q] T236A from transiently transfected COS1 cells produced the peak 2049.0336 $m/z$ corresponding to the theoretical $m/z$ value 2049.0518 expected for the mutated peptide without a phosphate. The sequence of this peptide was verified by MS/MS (Fig. 2A). The spectrum also contained a peak at 2129.0057 $m/z$ that corresponded to the theoretical $m/z$ value 2129.0181 expected for the mutated peptide with a phosphate. This peak was subjected to MS/MS and the resulting spectra confirmed phosphorylation at serine 239 (Fig. 2B). The absence of peaks at 2159.0287 $m/z$ and 2079.0623 $m/z$ and the presence of peaks at 2049.0336 $m/z$ and 2129.0057 $m/z$ demonstrate the shift in $m/z$ expected from mutation of threonine to an alanine. This data indicates that loss of a threonine did not affect the ability of the peptide to be phosphorylated and confirms the phosphorylation of serine 239. However, these data do not rule out the possibility that threonine 236 was phosphorylated. On the other hand, MALDI-TOF MS analysis of an in-gel chymotryptic digest of immuno-purified ataxin-1 [30Q] S239A from transiently transfected COS1 cells produced only the peak 2049.0336 $m/z$ formed by MALDI ionization of ataxin-1 S239A.

Fig. 3. MS/MS of ataxin-1 with serine 239 mutated to alanine produced a peptide corresponding to ataxin-1 without phosphate. Samples were prepared for MALDI-TOF MS/MS with a Millipore ZipTipC18 according to manufacturer’s protocol. Full scans of ataxin-1 T236A digested with chymotrypsin were collected from 500–3500 $m/z$ on a MALDI QSTAR XL quadrupole time-of-flight mass spectrometer. MS/MS was performed by gating on the peptide of interest, from immunoprecipitated material isolated from transfected cells and used in the full scan. All peaks labeled were three times above background. b and y ions are labeled above the corresponding peak in the spectrums. Degree signs denote the loss of a water and asterisks denote loss of NH3 from a fragment ion [14]. Immonium ions are labeled with an I and int indicates internal fragmentation. The data were plotted in GraphPad Prism using $m/z$ values that were smoothed and centroided in BioAnalyst (ABI). Expanded view of peak at 2063.0546 $m/z$ formed by MALDI ionization of ataxin-1 S239A. MS/MS ion spectrum of the peptide 2063.0546 $m/z$. The amino acid sequence is displayed above the spectrum and corresponds to the unphosphorylated peptide from residues 229–248 of ataxin-1 S239A.
2063.0546 m/z, corresponding to the theoretical m/z value 2063.0674 expected for the mutated peptide 229–248 with no phosphorylated residues (Fig. 3). This suggests that threonine phosphorylation may be less abundant or that the peptide containing phospho-threonine was not ionized. Even though mass spectrometry data obtained from the non-mutated ataxin-1 protein demonstrated phosphorylation of threonine 236 and serine 239, mass spectrometry of the mutated proteins could only support phosphorylation at site serine 239.

4. Discussion

Understanding the phosphorylation status of ataxin-1 undoubtedly will provide insight into the function that ataxin-1 plays within neurons. Posttranslational modifications such as phosphorylation have been shown to influence protein localization, protein–protein interaction and cellular signaling, all of which play a role in SCA1 pathogenesis. The novel phosphorylation site identified in this study, serine 239, and the possible phosphorylation of threonine 236 may play a role in protein localization and interaction, which may in turn influence the normal function or the mechanism of SCA1 disease.

It has been demonstrated that the expansion of the unstable CAG tract known to result in SCA1 causes down-regulation of genes involved in signal transduction and calcium homeostasis [11,12]. A recent publication demonstrated that ataxin-1 interacts with the transcriptional co-repressor that mediates silencing of retinoid and thyroid receptor signaling, SMRT. This study also showed that when ataxin-1 is tethered to DNA, it can invoke transcriptional repression [13]. Interestingly, serine 239 is within the consensus sequences targeted by CKI, Cdc2/Cdk5 and most precisely ERK (Fig 4A), a component of the MAP kinase pathway, known for activating transcription factors.

Residues threonine 236 and serine 239 lay only plus 10 and 13 amino acids away from the polyglutamine tract, respectively (Fig. 4B). Therefore, it is possible that altered conformation resulting from expansion of the polyglutamine tract may inhibit phosphorylation and/or protein–protein interaction at these residues. Examination of the effects of polyglutamine tract length on phosphorylation at sites 236 and 239 will be of interest.

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References

