Huntingtin Phosphorylation Sites Mapped by Mass Spectrometry: Modulation of Cleavage and Toxicity


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Huntington (Htt) is a large protein of 3144 amino acids, whose function and regulation have not been well defined. Polyglutamine (polyQ) expansion in the N-terminus of Htt causes the neurodegenerative disorder Huntington’s disease (HD). The cytotoxicity of mutant Htt is modulated by proteolytic cleavage with caspases and calpains generating N-terminal polyQ-containing fragments. We hypothesized that phosphorylation of Htt may modulate cleavage and cytotoxicity. In the present study, we have mapped the major phosphorylation sites of Htt using cell culture models (293T and PC12 cells) expressing full-length myc-tagged Htt constructs containing 23Q or 148Q repeats. Purified myc-tagged Htt was subjected to mass spectrometric analysis including matrix-assisted laser desorption/ionization mass spectrometry and nano-HPLC tandem mass spectrometry, used in conjunction with on-target alkaline phosphatase and protease digestions. We have identified more than six novel serine phosphorylation sites within Htt, one of which lies in the proteolytic susceptibility domain. Three of the sites have the consensus sequence for ERK1 phosphorylation, and addition of ERK1 inhibitor blocks phosphorylation at those sites. Other observed phosphorylation sites are possibly substrates for CDK5/CDC2 kinases. Mutation of amino acid Ser-536, which is located in the proteolytic susceptibility domain, to aspartic acid, inhibited calpain cleavage and reduced mutant Htt toxicity. The results presented here represent the first detailed mapping of the phosphorylation sites in full-length Htt. Dissection of phosphorylation modifications in Htt may provide clues to HD pathogenesis and targets for therapeutic development.

Huntington’s disease (HD) is a neurodegenerative disease caused by an elongated polyglutamine (polyQ) stretch in the huntingtin protein (Htt) and is characterized by involuntary movements, personality changes, dementia, and early death (1). Under non-pathological conditions, this stretch of glutamines ranges from 2 to 34 repeats. Repeat lengths of 36 or greater cause HD. The threshold of polyQ length for Htt aggregation is quite similar to the threshold for disease suggesting that a conformational change in Htt triggers the disease. However, it should be pointed out that the aggregates forming inclusions are not likely toxic themselves (2).

Htt is a protein with 3144 amino acids with a molecular weight of approximately 350 kDa. Its function and regulation are incompletely understood. Upon polyQ expansion in the N-terminus of Htt, a conformationally altered protein is produced. Structural predictions suggest that Htt is composed of four HH-HEAT domains with two unstructured proteolytic susceptibility domains (these areas contain PEST sequence) (Fig. 1). In addition Htt harbors a potential nuclear localization signal (NLS) at the end of the second HH2 domain and a confirmed nuclear export signal (NES) in the third HH3 domain (3).

Posttranslational modifications, especially phosphorylation and proteolytic cleavage, may facilitate the initial conversion of Htt from a normal to an abnormal conformation, and thus may be initiating steps in a pathogenic cascade. Thus understanding how Htt is posttranslationally modified in the context of polyQ expansion may give us insight into HD pathogenesis. Some posttranslational modifications of Htt have been described, such as ubiquinination (4) and SUMOylation (5). One important previous report has shown that phosphorylation of Htt at Ser-421 by Akt1 is neuroprotective against HD cellular toxicity (6,7).
Proteolysis of Htt has been well characterized and occurs in the proteolytic susceptibility region of Htt (Fig. 1). We have previously shown Htt is cleaved in several places by caspase enzymes (8,9) and cleavage contributes to toxicity (10,11). Caspase enzymes that can cleave Htt include caspase-2, -3, -6, and -7, and cleavage occurs between amino acids 513 and 587 (9,11,12). Htt is cleaved in several places by calpains as well (13,14) and this may also contribute to toxicity (15). The major calpain sites are located between amino acids 469 and 537 (13,15).

Systematic analysis of phosphorylation or other types of modifications of Htt protein and its consequences for Htt biochemistry and function have not previously been described. To identify posttranslational modifications in Htt, specifically phosphorylation, we generated myc-tagged full-length Htt23Q/148Q constructs for expression in 293T cells, PC12 cells and transgenics to use for our investigations (16). Htt was purified by immunoprecipitation using an anti-myc antibody, separated by 1D SDS PAGE and then subjected to proteolytic in-gel digestion and mass spectrometric analysis including MALDI-MS peptide mass fingerprinting (PMF) and tandem mass spectrometry (nano-HPLC-ESI-MS, MS/MS). In addition, a hypothesis-driven multiple stage mass spectrometry strategy was employed for phosphopeptide analysis using a vMALDI-LTQ mass spectrometer as described (17). This study describes the first extensive mass spectrometric sequence mapping and characterization of full-length Htt. During this work we identified six novel phosphorylation sites. We describe the biological significance of one of the phosphorylation sites in terms of proteolysis of Htt and cytotoxicity in cell culture.

**MATERIALS AND METHODS**

**Materials**—Reagents for protein chemistry including iodoacetamide and DTT were obtained from Sigma. Sequencing grade modified trypsin (porcine, Promega), chymotrypsin (bovine pancreas, Roche Molecular Biochemicals), and Asp-N protease (Pseudomonas fragi, Roche Molecular Biochemicals) were utilized for in-gel digestion reactions. HPLC solvents acetonitrile (ACN) and water were obtained from Burdick & Jackson. A matrix solution of α-cyano-4-hydroxycinnamic acid (33 mM) in ACN/methanol (Agilent Technologies) was used for MALDI-MS experiments. For vMALDI MS/MS, 2.5-dihydroxybenzoic acid (DHB; LaserBio Labs) in 50% ACN was used as matrix.

**Cell Culture and Transfection**—Full-length Htt constructs with polyQ repeats of either 23Q or 148Q (pTet-c-myc-FL23Q and pTet-c-myc-FL148Q) were generated containing N-terminal c-myc epitopes (pTet-splice vector, Life Technologies, Inc.) (16). pTet-c-myc-FL23Q and pTet-c-myc-FL148Q were transfected into 293T cells (10 cm plate, DMEM with 10% fetal bovine serum (FBS)) with Superfect (Qiagen) according to the manufacturer’s directions. Cells were harvested 48-72 h after transfection. In addition, a stable inducible PC12 cell model expressing full-length Htt (pTet-c-myc-FL148Q) was established as previously described (18). PC12 cells were grown in DMEM with 5% FBS, 10% horse serum, 100 µg/mL G418, 200 µg/mL hygromycin, 200 ng/mL doxycycline, 100 units/mL penicillin and 100 units/mL streptomycin. Full-length Htt148Q was expressed by removal of doxycycline. Cells used to obtain Htt for mass spectrometric analysis where cultured under conditions of differentiation in the presence of NGF (50 ng/mL) and low serum (0.5% FBS, 1% horse serum) for 8 days in the absence of doxycycline. Cells were cultured in serum-free media for 24 h prior to harvesting to reduce protein contamination for mass spectrometric analysis.

**Kinase Inhibitor Studies**—Kinase inhibitors U0126 (10 µM, 50 µM, Cell Signaling) and roscovitine (25 µM, 100 µM, 200 µM, Calbiochem) were solubilized in DMSO (Sigma) and added to cells 24 h after transfection for 24 h. Cells were treated with DMSO as a control. Similarly, lithium chloride (5, 25 mM) was added to cells with sodium chloride utilized as a control. Cells were harvested by scraping and centrifugation at 500 g. Immunoprecipitation and Western blot analysis on cell lysates were carried out as described below.

**Site-Directed Mutagenesis**—Site-directed mutagenesis of Htt constructs was performed using QuikChange kit (Stratagene) and the following primers: serine at amino acid 1201 was converted to an alanine, F 5′-CAACGATCTGTACCGTGGCCTCCAAGAAAGGCAGTGAGG-3′, R 5′-CCTACTGCTTTCCTTGGAGACCACCGGTACAGATGCTTG-3′; serine at amino acid 536 was converted to aspartic acid, F 5′-GGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-GGACGGCGCTGACCTGGCTGTGGTGTTGGCTCAAGATATCC-3′; serine at amino acid 535 was converted to aspartic acid, F 5′-GGAGGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-CGGTGGTGTTGGCTCAAGATATCC-3′; serine at amino acid 533 was converted to aspartic acid, F 5′-GGAGGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-GGTGGTGTTGGCTCAAGATATCC-3′; serine at amino acid 532 was converted to aspartic acid, F 5′-GGAGGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-GGTGGTGTTGGCTCAAGATATCC-3′; serine at amino acid 531 was converted to aspartic acid, F 5′-GGAGGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-GGTGGTGTTGGCTCAAGATATCC-3′; serine at amino acid 530 was converted to aspartic acid, F 5′-GGAGGATATTTGACGCAACAGGAGACGTCAGCGCGGTC-3′, R 5′-GGTGGTGTTGGCTCAAGATATCC-3′.

PCR was performed using 50 ng DNA, 5 µL 10X Pfu buffer (Stratagene), 0.2 mM dNTPs (Roche Molecular Biochemicals), 125 ng each of forward and reverse primers (Integrated DNA Technologies), 5% DMSO (Sigma) and 1 µL Pfu polymerase (Stratagene) at 96 °C for 1 min, 18 cycles at 96 °C for 50 s, 55 °C for 1 min and 68 °C for 24 or 45 min, and 68 °C for 7 or 10 min. Plasmids were DpnI
(Stratagene)-treated, transformed into XL1-Blue Supercompetent cells (Stratagene) and purified using the Qiagen Plasmid Mini Kit. Mutations, CAG repeat length and construct integrity were confirmed by DNA sequencing. Mutated constructs of different amino acid length were generated (Htt residues 1-1212 and full-length Htt including a myc-tag), and expressed as described above.

**Immunoprecipitation and Western Blot Analysis**—Harvested cells were lysed with M-PER (Mammalian Protein Extraction Reagent, Pierce) or RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 1% DOC, 1% NP40) with protease inhibitors (Mini Complete, Roche) and phosphatase inhibitors (1 mM NaFl, 1 mM Na3VO4). Lysates were sonicated and then spun to remove debris (16,000 g, 20 min). Protein concentration was determined with BCA Protein Assay kit (Pierce). Htt was immunoprecipitated overnight from cell lysates (500-2000 µg) using the ProFound Mammalian C-myc Tag IP/Co-IP kit (Pierce) following the manufacturer’s protocol with a final wash in 10 mM Tris pH 7.4 to remove excess salt and elution Protocol 2 with 25 µL non-reducing sample buffer (0.3 M Tris-HCl pH 6.8, 5% SDS, 50% glycerol, dye). Immunoprecipitated sample (3 µL for Western blot analysis or 22 µL for mass spectrometric analysis) was resolved by SDS-PAGE on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) or 22 µL for Western blot analysis (0.3 M Tris-HCl, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, pH 7.9). Samples were subjected to phosphatase treatment. Calf intestine alkaline phosphatase (1 µL, New England BioLabs) in 50 mM NH4CO3 (pH 8.0) was added on top of the dried analyte/matrix MALDI spot for 1 h at 37 °C as described in (22). After incubation, 1 µL matrix was added to recrystallize the sample. Alternatively, aliquots of the Htt digestions were incubated in solution with 1 µL alkaline phosphatase for 1-2 h in the presence of NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, pH 7.9). Samples were subsequently analyzed by MALDI mass spectrometry.

**Phosphatase Treatment**—Proteolyzed Htt samples were subjected to phosphatase treatment. Calf intestine alkaline phosphatase (1 µL, New England BioLabs) in 50 mM NH4CO3 (pH 8.0) was added on top of the dried analyte/matrix MALDI spot for 1 h at 37 °C as described in (22). After incubation, 1 µL matrix was added to recrystallize the sample. Alternatively, aliquots of the Htt digestions were incubated in solution with 1 µL alkaline phosphatase for 1-2 h in the presence of NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, pH 7.9). Samples were subsequently analyzed by MALDI mass spectrometry.

**In-gel Proteolytic Digestion of Proteins**—Proteins were digested manually to maximize sensitivity and efficiency. Protein bands were destained and dehydrated with ACN. Subsequently, proteins were reduced with 10 mM DTT in 25 mM NH4HCO3 at 56 °C for 1 h and alkylated with 55 mM iodoacetamide in 25 mM NH4HCO3 at RT for 45 min. For chymotrypsin digestion, 4 M guanidine hydrochloride/25 mM NH4HCO3 was used in the reduction step in order to fully digest Htt in the gel. Samples were incubated overnight with trypsin (125 ng, 37 °C), chymotrypsin (200 ng, 25 °C) or Asp-N (100-200 ng, 37 °C). The resulting proteolytic peptides were subjected to aqueous (100 µL H2O, sonication, 10 min) and hydrophobic extraction (50 µL 50% ACN/5% formic acid), and analyzed by mass spectrometry after concentration under vacuum to 10-15 µL final volume.

**Mass Spectrometry**—Mass spectra of digested protein gel bands were obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Voyager DESTR plus instrument (Applied Biosystems) and proteolytic peptide extracts were analyzed by reverse-phase nano-HPLC-MS/MS with an Ultimate HPLC (Dionex) connected to a QSTAR Pulsar I quadrupole orthogonal TOF mass spectrometer (MDS Sciex) as previously described (19,20). In addition, peptides obtained from Htt were analyzed by vacuum MALDI-MS, MS/MS, and MS3 on a vMALDI-LTQ™ linear ion trap (Thermo Electron, San Jose). For these experiments, samples were analyzed first by MS/MS for the neutral loss of phosphoric acid (98 Da) (17). Peptides showing this neutral loss were interrogated further by MS3 by selecting the (M + H – 98)+ peak as precursor. For further experimental detail using vMALDI-MSn for phospho-peptide analysis see Supplementary Material (21) and previous reports (17,21).

**Bioinformatics and Protein Database Searches**—Mass spectrometric data were analyzed with the bioinformatics database system RADARS (Genomic Solutions) (23), Mascot (Matrix Sciences, London) (24), and Sequest (25). Routinely, MALDI-MS data were analyzed with RADARS using the search engine ProFound for PMF, matching against peptides from known protein sequences as previously described (20). For ESI-MS/MS data sets, tandem mass spectra were submitted to the in-house licensed database search engine Mascot (version 2.1) (Matrix Sciences). A custom-designed protein database for human full-length Htt protein was constructed and incorporated into Mascot that could be searched more exhaustively for extended sequence coverage and identification of a more complete set of posttranslational
modifications. To extract and compile peak lists from ESI-MS data generated during an LC-MS/MS run, Mascot Distiller (1.1.1) and Distiller MDRO Software Developer’s Package (Matrix Sciences) were used in conjunction with an in-house Java program, “MS-Assign” (20). Therefore the generated ESI-MS peak lists were submitted to the Mascot search engine for PMF analysis at a mass accuracy of ± 50 ppm. MALDI-MS data were searched using Sequest (for details see Supplement). A Java program was developed in-house, “SeqDisp”, to graphically display the observed protein sequence coverage of peptides obtained from ESI-MS/MS, ESI-MS, and MALDI-MS data.

Toxicity Measurements— Caspase activity was measured using the ApoAlert Caspase-3 Fluorescent Assay Kit (Clontech, Palo Alto, CA) as described in our previous work (15).

RESULTS

Htt is Constitutively Phosphorylated— Htt is a challenging protein to analyze for posttranslational modifications with a molecular weight of approximately 350 kDa (3144 amino acid residues) and long polyQ stretches that are susceptible to aggregation. Further, human full-length Htt contains 307 serine residues, 170 threonine residues, and 62 tyrosine residues, many of which have predicted kinase consensus sequence motifs (Table 1).

In the present study, we expressed myc-tagged human full-length Htt constructs containing 23 and 148 polyQ repeats in 293T or PC12 cells. Htt23Q or Htt148Q cell lysates were immunoprecipitated using a myc-tag antibody and subsequently subjected to 1D SDS PAGE or Western blot analysis. Western blots of immunoprecipitated Htt23Q and Htt148Q were probed with different antibodies as shown in Fig. 2. The N-terminal Htt antibody BKP1 revealed immunoreactivity for Htt23Q and Htt148Q migrating at a molecular weight corresponding to the theoretical MW of full-length Htt of ~ 350 kDa (Fig. 2A). In addition, an anti-phospho-Ser-Pro antibody (16B4) was used that specifically recognizes phosphorylated serines that are adjacent to a proline residue. Htt23Q and Htt148Q were immunoreactive to this antibody (Fig. 2B, lanes 2 and 3) indicating that Htt was phosphorylated by proline-directed serine kinases. Htt23Q and Htt148Q were immunoreactive to an anti-phospho-Ser (1C8) antibody as well. This is a monoclonal antibody immunoreactive to a broad range of serine phosphorylated proteins, preferring positively charged amino acids directly neighboring the phosphoserine residues (Fig. 2C, lanes 2 and 3). These results suggest that we were able to isolate full-length human Htt that is endogenously phosphorylated by several different kinases during protein expression and these posttranslational modifications are constitutively present in Htt.

Htt is Constitutively Phosphorylated by MEK1 Kinases— Kinase inhibitor studies were performed to determine which group of kinases might be involved in Htt phosphorylation. 293T cells expressing Htt23Q and Htt148Q were treated with kinase inhibitors U0126 (MEK1/2 inhibitor), roscovitine (CDK5 inhibitor), and lithium chloride (GSK3 inhibitor). MEK1/2 inhibitor, U0126, decreased the overall level of Htt phosphorylation as detected by Western blots probed with an anti-phospho-Ser-Pro antibody (Fig. 3). Studies with roscovitine and lithium chloride, also using Western blot analysis and phosphoserine antibodies, were inconclusive. This is most likely due to the presence of several other abundant phosphorylation sites that are not affected by these kinase inhibitors (data not shown).

Immunopurification of Htt for Mass Spectrometry— In order to determine the unique sites of phosphorylation within Htt by mass spectrometry, we optimized the immunopurification of Htt using several different cell lysis buffers (Fig. 3; see Methods). Htt was immunoprecipitated from relatively large amounts of starting material (500 to 2000 µg of cell lysate per immunoprecipitation) from either 293T or PC12 cells. After release of Htt from the myc-tag antibody, the proteins were separated by 1D SDS PAGE and stained with the fluorescent dye Sypro Ruby (Fig. 4). Microgram quantities of Htt were obtained, sufficient for our analysis of posttranslational modifications by mass spectrometry.

Mass Spectrometric Characterization of Full-length Htt— To determine the sites of phosphorylation in Htt, we digested the Htt protein with trypsin, chymotrypsin, or Asp-N, and subjected the resulting peptides to both MALDI-MS and HPLC-ESI-MS/MS analysis. Mass spectrometry can give sequence data from the Sypro Ruby stained band or gel spot. The protein is digested with a protease in the gel, peptides are eluted and introduced into the mass spectrometer. The mass spectrometer determines the mass of the peptides and the sequence (by collisionally induced dissociation). From the masses of the peptide fragments, sequence data is determined by comparison with known sequences. Posttranslational modifications of these peptides can be identified by shifts in masses (i.e. 80 kDa for phosphorylation). As shown in Fig. 5A, protonated molecular ions, [M + H]+, for 31 peptides were detected by MALDI-MS (i.e., PMF) for Htt23Q after tryptic digestion, yielding an overall protein sequence coverage of 15%. Chymotryptic digestion of Htt23Q generated 68 peptides yielding an overall protein sequence coverage of 27% (Fig. 5B). We also digested Htt23Q with the protease Asp-N that cleaves N-terminal to Asp and Glu residues, and MALDI-MS analysis resulted in 13% sequence coverage (data not shown). Proteolytic samples were then subjected to an on-line separation by nano-HPLC reversed-phase C18 chromatography directly coupled to a hybrid quadrupole...
time-of-flight mass spectrometer (QSTAR). Peptides were selected for MS/MS and fragmented by collision-induced dissociation (CID) providing peptide sequence information. Analysis of the nano-HPLC-ESI-MS/MS with MALDI-MS gave good sequence coverage of Htt. A complete list of all Htt peptides that were sequenced and confirmed by mass spectrometry is provided in Supplementary Table S1 (for graphic display of Htt sequence coverage see Supplementary Fig. S1).

**Mass Spectrometric Identification of Phosphorylation Sites in Full-length Htt using Nano-HPLC-ESI-MS/MS**

To determine specific sites of phosphorylation within Htt, we searched the ESI-MS/MS spectra with Mascot using a custom-designed Htt database. With this analysis, we mapped six novel phosphorylation sites of Htt. The results are summarized in Table 2 and represent phosphorylation sites identified by both MALDI-MS and nano-HPLC-ESI-MS/MS (see Table S2 for details). Other types of Htt amino acid modifications, including oxidation and deamidation, are noted in Table S3.

As a representative example of how we assigned specific phosphorylation sites in Htt, Fig. 6 shows the tandem mass spectrum of monophosphorylated peptide EKEPGEQ-ASVPL$^{[520]}$PK (residues 1189-1203), generated from tryptic digestion of Htt23Q. A precursor ion [M + 2H]$^{[2+]}$ at m/z 838.41$^{[2+]}$ (M=1674.82 Da) was selected for MS/MS, and the fragmentation pattern was indicative of the presence of one phosphate group on serine residue 1201. A nearly complete series of y- ions and several b-ion fragments were observed, including several y$_{18}$-98 ions which result from the neutral loss of phosphoric acid (-H$_3$PO$_4$). The y-ion pairs observed at m/z 411.2/313.2 (y$_{11}$/y$_{11}$-98) and 621.3/523.3 (y$_{18}$/y$_{18}$-98) are indicative of the phosphorylation site at Ser-1201 as these fragment ions are shifted by 80 Da (mass of one phosphate group) compared to the corresponding fragments generated from the non-phosphorylated peptide. Alkaline phosphatase treatment on the MALDI target resulted in the disappearance of the diphosphorylated peptide with [M + H]$^{[+]}$ at m/z 2228.4 and in its mono- and diphosphorylated forms ([M + H]$^{[+]}$ at m/z 2308.4 and 2388.3, respectively) (Fig. 7A). The observed mass shifts of 80 and 160 Da correspond to the addition of one or two phosphate groups to the corresponding non-phosphorylated peptide.

To determine the site of phosphorylation within this specific peptide we performed online nano-HPLC-MS/MS. Tandem mass spectra (ESI-MS/MS) were obtained of the mono-phosphorylated peptide with [M + H]$^{[+]}$ at m/z 2444.7 due to dephosphorylation (Fig. 7B). The mono-phosphorylated peptide with [M + H]$^{[+]}$ at m/z 2308.4 decreased significantly in intensity while the non-phosphorylated peptide with [M + H]$^{[+]}$ at m/z 2228.4 increased. Observed peptides at m/z 2398.5 (residues 1527-1549, contained oxidized methionine) and at m/z 2444.7 (residues 110-131 or 1394-1415) were not affected by the phosphatase treatment and served as landmarks to compare relative peak abundances.

To determine the site of phosphorylation within this specific peptide we performed online nano-HPLC-MS/MS. Tandem mass spectra (ESI-MS/MS) were obtained of the mono- and diphosphorylated form of the peptide DAPAPSS$^{[563]}$PPTS$^{[567]}$P-VNSRKHAGV (residues 2647-2668) were detected in its non-phosphorylated form ([M + H]$^{[+]}$ at m/z 2228.4) and in its mono- and diphosphorylated forms ([M + H]$^{[+]}$ at m/z 2308.4 and 2388.3, respectively) (Fig. 7A). The observed mass shifts of 80 and 160 Da correspond to the addition of one or two phosphate groups to the corresponding non-phosphorylated peptide. Alkaline phosphatase treatment on the MALDI target resulted in the disappearance of the diphosphorylated peptide with [M + H]$^{[+]}$ at m/z 2228.4 and 2308.4. Decreased significantly in intensity while the non-phosphorylated peptide with [M + H]$^{[+]}$ at m/z 2228.4 increased. Observed peptides at m/z 2398.5 (residues 1527-1549, contained oxidized methionine) and at m/z 2444.7 (residues 110-131 or 1394-1415) were not affected by the phosphatase treatment and served as landmarks to compare relative peak abundances.

In addition, we mutated a number of the identified phosphorylation sites and recorded the mass spectra. As a representative example, the ESI-MS/MS spectrum of peptide EPGEQASVPLA$^{[120]}$PK (residues 1191-1203) obtained after tryptic digestion of Htt23Q demonstrated the successful site-directed mutagenesis from Ser-1201 to Ala-1201. The molecular ion [M + 2H]$^{[2+]}$ at m/z 661.85$^{[2+]}$ (M=1321.68 Da) was selected for CID (data not shown). The corresponding phosphopeptide containing pSer-1201 previously observed in wild-type Htt was not detected in this Htt23Q S1201A (data not shown). In addition, we identified Ser-1181 as a unique phosphorylation site after tandem ESI-MS/MS analysis of the tryptic peptide A$^{[1169]}$ALPSLTNPSP$^{[118]}$PIR (Fig. 9).
Several other novel phosphorylated peptides generated after proteolytic digestion of Htt protein were identified by tandem mass spectrometry as summarized in Table 2. An ESI-MS/MS spectrum was recorded for monophosphorylated peptide DSLSpS967PPVSHPL (residues 2071-2084) as shown in Supplementary Fig. S2. The molecular ion [M + 2H]^{2+} at m/z 750.38±2 (M=1498.74 Da) was selected for CID, and a series of y-ions, (y_{9}/y_{8}/P)-ion pairs and several b-fragment ions were observed that indicated the phosphorylation of Ser-2076 (see Supplementary Fig. S2).

We also identified the phosphorylated peptide T^{91}AAKEESGGRSRSGpS^{621}VEL^{625} by tandem mass spectrometry containing a single phosphorylation site at Ser-421. The molecular ion, [M + 3H]^{3+} at m/z 672.0^{±6} (M=2012.98 Da) was selected for CID (see Supplementary Fig. S3). pSer-421 was the first phosphorylation site of Htt described and was identified using biochemical and molecular methods (6,7).

**Hypothesis-Driven Investigation of Phosphorylation Sites in Htt Using Neutral Loss Scanning Mass Spectrometry**– To investigate Htt for additional phosphorylation sites we adapted a hypothesis-driven multiple-stage mass spectrometric approach originally described by Chang et al. (17). Analysis was performed on a Finnigan™ LTQ™ vMALDI quadrupole linear ion trap. Briefly, peptides with highly predicted kinase consensus sites were examined using vMALDI-MS/MS for the presence (or absence) of phosphoserine and phosphothreonine residues based on the preferential neutral loss of phosphoric acid (-98 Da). Subsequent MS^3 analysis of the corresponding (M + H - 98)^+ peaks provided sequence data to confirm or reject the hypothetical assignments. The phosphorylation sites that were identified by ESI-MS/MS in this study were also investigated as positive controls. An example is displayed in Fig. 9A and 9B showing tandem mass spectra of peptide AALPSLTNPPSLpS^{1181}PIR obtained from Htt23Q and Htt148Q, respectively. In both cases, a loss of 98 Da from the [M + H]^+ precursor ion at m/z 1713.7±7 (M=1712.7) was observed to yield an abundant neutral loss of 98 Da at m/z 1615.8. This peptide was selected for MS^3 analysis (Fig. 9C), revealing a characteristic set of sequence ions (y_{9}/y_{8}-17, y_{0,11}, and y_{13}) that confirmed the phosphorylation site as p-Ser-1181. Supplementary Fig. S5-S10 shows additional vMALDI MS/MS and MS^3 spectra of peptides containing the phosphorylation sites pSer-2653, pSer-2657, pSer-1181, pSer-1201, and pSer-421, confirming our results.

Using the hypothesis-driven approach, we also investigated a predicted set of Htt peptides containing Ser/Thr sites that had been identified as potential kinase consensus motifs with high prediction scores (see Table 1 and Table S4). However, no new phosphorylation sites were identified from these additional analyses.

**Mass Spectrometric Identification of a Phosphorylation Site in the Proteolytic Susceptibility Domain of Htt**– We detected another novel monophosphorylated peptide by LC-MS/MS, phospho-[DILS^{633}HS^{555}S^{677}QVAVPS], corresponding to Htt residues 530-544 obtained from an Asp-N protease digest. As shown in Supplementary Fig. S4, a tandem mass spectrum was recorded for the monophosphorylated peptide with a [M + 2H]^{2+} at m/z 797.40±2 (M=1592.78 Da), 80 Da higher than the non-phosphorylated peptide counterpart at m/z 757.40±2 (M=1512.78 Da). This particular phosphorylation site lies in the proteolytic susceptibility domain of Htt, and our previous work has identified amino acid 536 as a site of calpain cleavage in Htt (15). The peptide fragmentation patterns could not uniquely determine which of the serines is phosphorylated (Ser-533, Ser-535, or Ser-536).

**Biological Significance of Phosphorylation at amino acid 536 of Htt**– One of the more interesting phosphorylation sites identified in our studies lies in the putative proteolytic susceptibility domain of Htt (PEST sequence) (Fig. 1). We have previously shown that calpains cleave Htt to produce N-terminal cleavage products (13,15). In those studies, we found calpain cleaves Htt15Q (1-1212) to yield a 72 kDa N-terminal product, and the site of cleavage was eliminated by mutation at the 536 amino acid region of Htt. This would suggest that the phosphorylation site identified in our current studies at amino acid 536 controls proteolysis of Htt by calpains. To test this, we mutated Ser-536 to aspartic acid to mimic phosphorylation (Fig. 10A,B). We found that Htt15Q or Htt138Q (1-1212) S536D, when expressed in 293T cells, was resistant to calpain cleavage. Production of the 72 kDa calpain-derived Htt fragment (92 kDa for Htt138Q (1-1212)) was eliminated (Fig. 10D). Mutation of serine to aspartic acid at amino acid 533 or 535 did not alter cleavage (Fig. 10A). The remaining immunoreactivity is due to cleavage of Htt at amino acid 513 by caspases (data not shown). We also evaluated whether calpain cleavage of polyQ-expanded Htt influenced toxicity through phosphorylation of this site. We expressed the calpain-resistant Htt15Q or 138Q (1-1212) S536D constructs in 293T cells and evaluated cytotoxicity. As shown in Fig. 10B, mutation of Ser-536 to aspartic acid to mimic phosphorylation, reduced cellular toxicity as measured by caspase activation and correlated with decreased proteolysis of mutant Htt.

**Kinase Consensus Sites for Htt**– We have identified six novel Htt phosphorylation sites in Htt23Q and Htt148Q expressed in 293T cells. These include pSer-536, pSer-1181, pSer-1201, pSer-2076, pSer-2653, and pSer-2657 (Table 2). Knowing the corresponding kinases for these


The phosphorylation site at Ser-536 which is further details regarding predictions see Supplementary Table S4. The phosphorylation site at Ser-2657 is predicted as a highly likely ERK1 substrate (ERK1 is a member of the MAP kinase pathway) (33). Phosphorylation sites at Ser-2653 is predicted as a highly likely ERK1 substrate (ERK1 is a member of the MAP kinase pathway) (33). Phosphorylation sites at Ser-2657 and Ser-2076 are predicted as ERK1 substrates using ScanSite prediction programs (using lower stringency searches) or substrates for other Pro-directed kinases (for further details regarding predictions see Supplementary Table S4). The phosphorylation site at Ser-536 which is discussed above does not contain a known kinase consensus site.

DISCUSSION

This study presents the first detailed mass spectrometric characterization of expressed Htt including mapping of the protein sequence by tandem mass spectrometry and by PMF. The major focus of this work was to apply mass spectrometric techniques to Htt protein characterization and identification of phosphorylation sites in Htt. This was accomplished by analyzing multiple protease digests of Htt by HPLC ESI-MS/MS as well as targeted neutral loss experiments using tandem vMALDI ion trap experiments of potential or hypothetical phosphorylation sites suggested by mass data or kinase prediction algorithms. These newly identified phosphorylation sites may be involved in the normal function of Htt as well as HD pathogenesis. Our current study provides a set of tools to investigate the role of posttranslational modifications of Htt in HD. We were able to map phosphorylation sites common to both wild-type (23Q repeat) and mutant (148Q repeat) Htt protein expressed in 293T and PC12 cells (Table 2, Table S5). Given the 42% sequence coverage obtained by ESI-MS/MS, and an overall 88% sequence coverage of Htt additionally considering PMF (ESI-MS and MALDI-MS) in our studies, it is likely we have identified the major constitutive phosphorylation sites in Htt. The methods employed in our studies, however, did not quantify the absolute level of the individual phosphorylation sites in Htt.

In addition to the data presented here, we also carried out a comprehensive analysis of two tryptic peptide digests of full length Htt after selective enrichment of phosphopeptides using immobilized metal ion affinity chromatography (IMAC) as described in the Supplementary Material section (34). Although we were able to confirm several phosphorylation sites already identified, no additional sites were detected.

Fig. 1 displays the location of the phosphorylation sites within Htt identified in this study. Htt contains 4 major HEAT domains indicated as HH1-HH4, several known proteolytic susceptibility domains, and a potential NLS. The identified novel phosphorylation sites are distributed throughout the protein sequence, i.e., pSer-536 in the proteolytic susceptibility domain, pSer-1181 and pSer-1201 in proximity to a potential Htt NLS (35,36), pSer-2076 within HEAT domain 3 (HH3), and pSer-2653 and pSer-2657 overlapping with a predicted C-terminal proteolytic susceptibility domain as well as a predicted calcineurin binding motif.

Kinase inhibitor studies with U0126, a MEK1/2 inhibitor, confirmed the phosphorylation of Htt protein by either MEK1/2 or ERK1 kinases (ERK1 is a kinase downstream of MEK1/2 in the MAP kinase pathway and would be equally inhibited upon U0126 inhibitor treatment). Htt phosphorylation sites pSer-2076, pSer-2653, and pSer-2657 are predicted to be phosphorylated by ERK1 kinase consistent with decreased levels of Htt phosphorylation with U0126 treatment (Fig. 3). U0126 treatment did not completely eliminate phosphorylation of all serines adjacent to prolines, consistent with other types of Htt phosphorylation sites. pSer-1181 and pSer-1201 are likely CDC2/CDK5 kinase consensus sites and should not be affected by MEK1/2 inhibitor treatment.

It has been suggested that proteolytic cleavage of mutant Htt may play an important role in the pathophysiology of HD. We have previously shown that the toxicity of caspase-resistant or calpain-resistant expanded Htt was markedly reduced in transfected cells (15). This suggests that posttranslational modifications that prevent the production of toxic polyQ-containing products would be neuroprotective. In the present study we identified a phosphopeptide, (D<sup>530</sup>IL<sup>532</sup>S<sup>533</sup>H<sup>535</sup>S<sup>536</sup>S<sup>537</sup>SQVSAVPS<sup>544</sup>)<sub>34</sub>, by ESI-MS/MS that is located within an N-terminal proteolytic susceptibility domain targeted by caspases and calpains (8,9,11,13,15,37). This region of Htt is particularly important because it contains both the calpain and the caspase cleavage sites of Htt. We have recently demonstrated that cleavage at amino acid Ser-536 is mediated by calpains (15). Functional analysis of this site (Fig. 10) suggests that phosphorylation of this site blocks cleavage by calpains and modulates toxicity of mutant Htt. The site of phosphorylation in the sequence DIL<sup>530</sup>IL<sup>532</sup>S<sup>533</sup>H<sup>535</sup>S<sup>536</sup>SQVSAVPS of Htt does not contain a known kinase consensus site. Future work will be directed at identifying the kinase responsible for phosphorylating Htt at this site.

Our findings demonstrate that phosphorylation of Htt at Ser-536 decreases Htt cellular cytotoxicity and the
production of cytotoxic fragments by modifying a site of calpain cleavage in Htt. Other work on Htt as well as the androgen receptor has shown that phosphorylation at more distal sites can modulate cleavage. In a related polyglutamine disease, Kennedy’s disease, we have shown that the phosphorylation of androgen receptor modulates proteolysis and toxicity (38). Our studies found that phosphorylation at Ser-514 of the androgen receptor, a site distal to the caspase cleavage site at amino acid 154, enhanced cellular toxicity and the production of cytotoxic fragments. Recent work on Htt suggests that CDK5-mediated phosphorylation of Ser-434 reduces cleavage of Htt at a distal caspase-3 cleavage site (amino acid 513) (39). A key role for phosphorylation of Ser-776 of ataxin-1 was shown to trigger toxicity in SCA1 in vivo (40,41). In this case protein interactions and ataxin-1 localization are altered by phosphorylation and not cleavage of the protein.

In summary, we have mapped the major constitutive phosphorylation sites of Htt and identified a critical phosphorylation event modulating Htt cleavage and toxicity. Future studies will be directed at understanding how these phosphorylation events affect the function and regulation of Htt and what their role is in HD.

Acknowledgments— Financial support was contributed by NIH grant NS40251A and HighQ (to L.M.E., B.W.G.), NIH postdoctoral fellowship F32 NS043937 (to J.G.) and NIH grants NS 16375 and 38144, the HDSA and HDF (to C.A.R.). We thank Thermo Electron Corporation for providing Finnigan™ vMALDI-LTQ™ linear ion trap mass spectrometer for evaluation purposes, and Dr. Rosa Viner for experimental advice. We thank Chip Witt for bioinformatics support and Chris Yoo for experimental support. We thank Michael Hayden for providing BKPI antibody to Htt.
FIGURE LEGENDS

Fig. 1. Schematic of Htt structure with four HEAT repeat domains (HH) with the indicated number of HEAT repeats and the caspase/calpain domain of Htt. Within the sequence we indicated regions of a putative aspartic endopeptidase site, unstructured proteolytic susceptibility domains (caspase/calpain), and a nuclear export site (NES). The red arrows indicate phosphorylation sites identified in these studies.

Fig. 2. Immunoprecipitation and Western blot analysis of Htt with anti-Htt and phospho-specific antibodies. Myc-tagged full-length Htt constructs (Htt23Q and Htt148Q) were expressed in 293T cells. Western blot of immunoprecipitated Htt23Q and Htt148Q probed with (A) polyclonal N-terminal Htt antibody BKP1, (B) monoclonal anti-phospho-Ser-Pro 16B4, and (C) monoclonal anti-phospho-Ser1C8 antibody.

Fig. 3. MEK1/2 inhibitor (U0126) decreases Htt phosphorylation. 293T transfected cells were treated with U0126 kinase inhibitor (10 µM, 24 h). Western blot of input cell lysates was probed with a N-terminal anti-Htt antibody (BKP1). Western blot of anti-myc immunoprecipitated Htt was probed with anti-Htt BKP1 antibody to show comparable protein expression levels for the different kinase inhibitor treatments, and a decrease in phosphorylation was detected with anti-phospho-Ser-Pro antibody (16B4) in the presence of U0126.

Fig. 4. Immunoprecipitation and 1D SDS-PAGE of Htt. Myc-tagged full-length Htt expressed in 293T or PC12 cells was immunoprecipitated from cellular lysates using the Pierce ProFound mammalian C-myc tag IP/Co-IP kit in M-PER buffer. Samples were separated by 1D SDS-PAGE on 4-12% Bis-Tris gels. Gels were stained with fluorescent Sypro Ruby protein gel stain (Invitrogen). The lower panel demonstrates that the expanded form of Htt immunoprecipitates at higher levels in a buffer composed of 20% RIPA and 80% M-PER (labeled as RIPA). Gel bands at approximately 50 kDa correspond to immunoglobulin. Protein samples were analyzed by mass spectrometry after in-gel digestion. Arrows indicate identified Htt protein.

Fig. 5. MALDI-TOF mass spectra of immunoprecipitated Htt23Q. The MALDI-MS TOF spectra display molecular ions [MH]+ of peptides obtained after in-gel proteolytic digestion of immunoprecipitated Htt23Q. (A) Het PMF resulting from trypptic digestion. An overall protein sequence coverage of 15% was obtained. (B) Htt PMF resulting from chymotryptic digestion. An overall protein sequence coverage of 27% was obtained.

Fig. 6. ESI-MS/MS spectrum of monophosphorylated peptide EKEPGEQAVPLpS1201PK (residues 1189-1203) obtained after trypptic digestion of Htt23Q. The molecular ion [M + 2H]+ at m/z 838.412+ (M=1674.82 Da) was selected for CID. Series of y-fragment ions and several b-fragment ions were observed; several y-ions featured loss of 98 Da due to loss of phosphoric acid.

Fig. 7. MALDI mass spectra displaying molecular ions, [M + H]+ of PMF obtained after Asp-N digestions of Htt23Q. (A) Peptide DAPAPSS2653PPTSP27P1PVNSRKHRAGV (residues 2647-2668) present in its non-phosphorylated form, [M + H]+ at m/z 2228.4, and in its mono- and di-phosphorylated forms, [M + H]+ at m/z 2308.4 for DAPAPSPS2653PPTSPPVNSRKHRAGV and [M + H]+ at m/z 2388.3 for DAPAPSPS2653PPTSPS2675PVNSRKHRAGV. (B) The same sample after incubation with alkaline phosphatase for 1 h.

Fig. 8. ESI-MS/MS tandem mass spectra of mono- and diphosphorylated peptides obtained after Asp-N digestion of immunoprecipitated Htt23Q. (A) Monophosphorylated peptide DAPAPSPS2653PPTSPVNSRKHRAGV (residues 2647-2668). The molecular ion [M + 3H]+ at m/z 770.072+ (M=2307.20 Da) was selected for CID. Series of y-fragment ions and several b-fragment ions were observed; several y-ions featured loss of -98 Da due to loss of phosphoric acid, proving serine phosphorylation on residue Ser-2653. (B) Diphosphorylated peptide DAPAPSPS2653PPTSPS2675PVNSRKHRAGV (residues 2647-2668). The molecular ion [M + 3H]+ at m/z 796.742+ (M=2387.20 Da) was selected for CID. The peptide fragmentation pattern proves one phosphorylation site at Ser-2653, and suggests the second phosphorylation site to be either Ser-2657 or Thr-2656, with Ser-2657 being the more likely site according to predictions.

Fig. 9. vMALDI-MS/MS and MS3 identification of phosphorylated peptide AALPSLTNPSSpS181PIR (residues A-1169 to R-1184) from Htt23Q and Htt148Q for pSer-1181. (A) The precursor ion [M + H]+ at m/z 1713.77+ (M=1712.7) was selected for MS/MS analysis from Htt23Q and (B) Htt148Q digests. In both cases, a strong neutral loss of 98 Da was observed due to loss of phosphoric acid, yielding an abundant fragment ion at m/z 1615.8 (A) and 1615.7 (B), respectively. In Panel (A) a
shorter collision energy activation time of 10 msec (instead of 30 msec) was used. (C) The neutral loss fragment ion at m/z 1615.8 was isolated and collisionally activated to yield the resulting confirmatory MS3 spectrum where pSer-1181 is now dehydroalanine.

**Fig. 10.** (A) Western blot of Htt (1-1212) expressed in 293T cells probed with a N-terminal Htt antibody. Mutation of Ser-536 to Asp dramatically decreases cleavage while the same mutation at Ser-535 or Ser-533 does not. (B) Htt138Q (1-1212) S536D mutation decreases toxicity in 293T cells while mutation S535D has no effect (**p < 0.005).

**Supplementary material:**

Tables and Figures are presented in a separate PDF file.
REFERENCES

Figure 1
Figure 2
<table>
<thead>
<tr>
<th></th>
<th>pcDNA3</th>
<th>Htt23Q</th>
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<tr>
<td>U0126</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>IP: Anti-myc</td>
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</table>

![Image of gel blots with antibody labels: Anti-Huntingtin, Anti-Phospho-Ser-Pro](image)

Figure 3
Figure 4
Figure 5

A

Trypsin-Htt

B

Chymotrypsin-Htt

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
**Figure 10**

A

![Western blot analysis showing monoclonal BKP1 antibody detection of tubulin and Htt138Q and Htt15Q proteins in pcIneo and Htt15Q (1-1212) and Htt138Q (1-1212) expression constructs.]

**B**

![Bar graph showing DEVDase activity/mg protein for various constructs: pcIneo, Htt15Q (1-1212), Htt15Q (1-1212) S536D, Htt15Q (1-1212) S535D, Htt138Q (1-1212), Htt138Q (1-1212) S536D, and Htt138Q (1-1212) S535D.]

The bar graph shows a significant increase in DEVDase activity for Htt138Q (1-1212) S536D compared to other constructs, indicated by the double asterisk symbol (**) on the graph.
Table 1. Predicted Phosphorylation Sites in Human Htta

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>Score</th>
<th>Prediction Percentile</th>
<th>Kinaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basophilic serine/threonine kinase group (Baso_ST_kin)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GGRSRSG-S-IVELIAG</td>
<td>S421</td>
<td>0.3742</td>
<td>0.030%</td>
<td>AKT1</td>
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<td>NLKSSSP-T-IRRTAAG</td>
<td>T267</td>
<td>0.3950</td>
<td>0.167%</td>
<td>PRKCA</td>
</tr>
<tr>
<td>AHHLLKNM-S-HCRQPSD</td>
<td>S636</td>
<td>0.4076</td>
<td>0.126%</td>
<td>PRKCM</td>
</tr>
<tr>
<td>LDRFRLS-T-MQDSLSP</td>
<td>T2068</td>
<td>0.4476</td>
<td>0.104%</td>
<td>AKT1</td>
</tr>
<tr>
<td>TSSTTRAL-T-FGCEAL</td>
<td>T1024</td>
<td>0.4817</td>
<td>0.180%</td>
<td>AKT1</td>
</tr>
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<tr>
<td>LVMEQEES-PPEEDTE</td>
<td>S2489</td>
<td>0.3843</td>
<td>0.130%</td>
<td>CSNK2B</td>
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<tr>
<td>ASSPPT-S-PVNSRKH</td>
<td>S2657</td>
<td>0.4047</td>
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<td>GSK3A</td>
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<tr>
<td>KAALPL-T-NPPSLSP</td>
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<td>0.4633</td>
<td>0.146%</td>
<td>GSK3A</td>
</tr>
<tr>
<td>MQDSLSP-S-PPVSSH</td>
<td>S2076</td>
<td>0.4759</td>
<td>0.184%</td>
<td>GSK3A</td>
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<tr>
<td><strong>Proline-dependent serine/threonine kinase group (Pro_ST-kin)</strong></td>
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</tr>
<tr>
<td>EVVAAPG-S-PYHRLLT</td>
<td>S3126</td>
<td>0.3267</td>
<td>0.054%</td>
<td>CDK5</td>
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<tr>
<td>LTNPPSL-S-PIRRKGK</td>
<td>S1181</td>
<td>0.3343</td>
<td>0.062%</td>
<td>CDK5</td>
</tr>
<tr>
<td>WWAEVQQ-T-PKRHLNL</td>
<td>T1859</td>
<td>0.3483</td>
<td>0.080%</td>
<td>CDK5</td>
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<tr>
<td>EVVAAPG-S-PYHRLLT</td>
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<tr>
<td>EQASVPL-S-PKGGSEA</td>
<td>S1201</td>
<td>0.3718</td>
<td>0.122%</td>
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<td>LTNPPSL-S-PIRRKGK</td>
<td>S1181</td>
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<td>QPGEQLL-S-PERRTNT</td>
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<td>0.3997</td>
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<td>ADAPAPS-S-PPTPVNN</td>
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<tr>
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<td>S1201</td>
<td>0.4438</td>
<td>0.195%</td>
<td>CDC2</td>
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<tr>
<td>STMQDSL-S-PSSPVSS</td>
<td>S2074</td>
<td>0.4486</td>
<td>0.171%</td>
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<td><strong>Phospho-serine/threonine binding group (sST_bind)</strong></td>
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<tr>
<td>ESGGRSR-S-GSIVELI</td>
<td>S419</td>
<td>0.4473</td>
<td>0.712%</td>
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<tr>
<td>VTTSSKS-S-LGFSYHL</td>
<td>S1228</td>
<td>0.4544</td>
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<tr>
<td>EHYRIN-T-LGWTSR</td>
<td>T2457</td>
<td>0.4575</td>
<td>0.807%</td>
<td>YWHAZ</td>
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<td><strong>Phospho-tyrosine binding group (Y_kin)</strong></td>
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<tr>
<td>LDGTDNQ-Y-LGLQIGO</td>
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<td>0.4056</td>
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<td>GALHGVLY-VLIECDLL</td>
<td>Y2790</td>
<td>0.4905</td>
<td>0.972%</td>
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<tr>
<td>ESFFSKL-Y-KVPLDTT</td>
<td>Y737</td>
<td>0.4100</td>
<td>0.293%</td>
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<td>PRLQLEL-Y-KEIKKNG</td>
<td>Y173</td>
<td>0.4587</td>
<td>0.546%</td>
<td>FYN</td>
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<td><strong>Protein Kinase A, PKC epsilon</strong></td>
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<td>TRFGRKL-S-IIRGIVE</td>
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<td>0.3627</td>
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<tr>
<td>QQTPKRH-S-LSTTKKL</td>
<td>S1864</td>
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<td>0.557%</td>
<td>PRKACG</td>
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<tr>
<td>APKSLRS-S-WASEEEA</td>
<td>S1110</td>
<td>0.4311</td>
<td>0.569%</td>
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<tr>
<td>AALGML-T-CMYTGKE</td>
<td>T2926</td>
<td>0.4478</td>
<td>0.366%</td>
<td>PRKCM</td>
</tr>
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</table>

(a) See Yaffe et al for predictions (31).
(b) Kinase sites searched and abbreviations: Akt kinase, AKT1; PKC alpha/beta/gamma, PRKCA; PKC mu, PRKCM; Casein kinase 2, CSNK2B; GSK3 kinase, GSK3A; Cdk5 Kinase, CDK5; Cdc2 kinase, CDC2; Erk1 kinase, EPHB2; 14-3-3 Mode, YWHAZ; Fgr kinase, FGR; Itk SH3, ITK; Crk SH3, CRK; Fyn SH2, FYN; Protein kinase A, PRKACG; PKC epsilon, PRKCE.
Table 2. Huntingtin phosphorylation sites identified by mass spectrometry

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<thead>
<tr>
<th>Obs. proteolytic phospho-peptide</th>
<th>Residue</th>
<th>Kinase</th>
<th>Mascot Score</th>
<th>SEQUEST Xcorr Value</th>
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<tr>
<td>TAAKEESGGRRS5pSIVEL</td>
<td>421</td>
<td>AKT-1</td>
<td>14</td>
<td>--</td>
</tr>
<tr>
<td>phospho-(DILSHSSSQVSAVPS)</td>
<td>533/536</td>
<td>--</td>
<td>41</td>
<td>--</td>
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<tr>
<td>AALPSTpNPPSLpSPK</td>
<td>1181</td>
<td>CDC2/CDK5</td>
<td>32</td>
<td>2.08 (MS2)</td>
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<tr>
<td>EKEpGEQASVPLpSPK</td>
<td>1201</td>
<td>CDC2/CDK5</td>
<td>58</td>
<td>3.29 (MS2)</td>
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<tr>
<td>GKEpKGEQASVPLpSPK</td>
<td>1201</td>
<td>CDC2/CDK5</td>
<td>13</td>
<td>4.23 (MS3)</td>
</tr>
<tr>
<td>EQASVLPspPKKGGEASAAPHRQSPK</td>
<td>1201</td>
<td>CDC2/CDK5</td>
<td>51</td>
<td>--</td>
</tr>
<tr>
<td>DSLSpPSPPVSHPPL</td>
<td>2076</td>
<td>ERK1</td>
<td>22</td>
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<tr>
<td>DAPSapSPPTpSVNSRHAGV</td>
<td>2653</td>
<td>ERK1</td>
<td>46</td>
<td>1.52 (MS2)</td>
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<tr>
<td>DAPSap8PPTp8PVNSRHAGV</td>
<td>2653</td>
<td>ERK1</td>
<td>44</td>
<td>2.37 (MS2)</td>
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<tr>
<td></td>
<td>2657</td>
<td>Pro-dir.</td>
<td>23</td>
<td>2.37 (MS2)</td>
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* Further mass spectrometric details are in Table S2.

a All phospho-peptides were confirmed by tandem mass spectrometry.

b Mascot scores of individual peptides that were observed by nano-LC ESI-MS/MS were determined as described in Perkins et al. (24).

c SEQUEST Xcorr value of individual peptides that were observed by MALDI-MS' (MS/MS') were determined as described in Eng et al. (25). The abundant neutral loss fragment ion (MH+ - 98) observed in MS2 spectra of the phospho-peptides was subjected to further fragmentation (MS3).

d Phosphorylation site: Ser-413, Ser-417, Ser-419 or Ser-421 (based on ESI-MS/MS); phospho-Ser-421 was previously identified as P-site in Htt.

e Phosphorylation site: Ser-533, Ser-535, or Ser-536 (based on ESI-MS/MS).

f Phosphorylation site: Ser-2657 or Thr-2656, with higher prediction score for Ser-2657.
Huntingtin phosphorylation sites mapped by mass spectrometry: Modulation of cleavage and toxicity
Birgit Schilling, Juliette Gafni, Cameron Torcassi, Xin Cong, Richard H. Row, Michelle A. LaFevre-Bernt, Michael P. Cusack, Tamara Ratovitski, Ricky Hirschhorn, Christopher A. Ross, Bradford W. Gibson and Lisa M. Ellerby

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