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Phosphoproteomic analysis of human brain by calcium phosphate precipitation and mass spectrometry

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Abstract

Alzheimer's disease (AD), the most common form of dementia, is manifested in the brain by the aggregation of amyloid plaques and neurofibrillary tangles. The tangles are primarily composed of microtubule-associated protein tau that is aberrantly hyperphosphorylated, suggesting that deregulated phosphorylation may contribute to AD pathogenesis. However, systematic analysis of the phosphoproteome in AD brain tissues has not been reported. We used calcium phosphate precipitation to analyze an AD postmortem brain, followed by liquid chromatography-tandem mass spectrometry. The protein sample was first resolved by one-dimensional polyacrylamide gel electrophoresis and subjected to gel excision and in-gel digestion. Phosphopeptides in the resulting peptide mixtures were enriched in a single step of calcium phosphate precipitation, and then analyzed by the LC-MS/MS approach. After database search, stringent filtering, and manual validation of neural loss in the MS/MS spectra, a total of 466 phosphorylation sites on 185 proteins including tau were identified. The majority of sites were not described previously. This study demonstrates the feasibility of combining calcium phosphate precipitation with mass spectrometry for phosphoproteome analysis of postmortem human brain tissue.

Keywords

Alzheimer's disease; protein phosphorylation; immobilized metal-affinity chromatography; calcium phosphate precipitation; proteomics

Introductions

Alzheimer's disease (AD) is the most common neurodegenerative disease, affecting more than 20 million patients worldwide^{1, 2}. The brains of AD patients are characterized by two pathological hallmarks: senile plaques composed of amyloid- β and neurofibrillary tangles composed of the microtubule-associated protein tau^{1, 3}. Accumulated evidence suggests that

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Supporting Information Available

TableS1 shows the list of phosphopeptides with protein accession number, description, peptide sequence, phosphorylation sites, XCorr score, ΔC_n , and charge states. XCorr scores are hyperlinked to our lab server to display the Sequest matching results. This information is available free of charge via the Internet at <http://pubs.acs.org>.

a key step in AD pathogenesis involves tau hyperphosphorylation that leads to the formation of characteristic neurofibrillary tangles^{4, 5}. Aberrant phosphorylation of several other proteins such as neurofilaments⁶ and microtubule-associated protein 1B (MAP1B)⁷ have also been found to associate with AD pathogenesis. These substrates may be modified by a number of kinases, such as GSK-3 β ⁸, cyclin-dependent kinase 5 (CDK5)⁸ and microtubule-affinity regulating kinase (MARK)⁸. Tau may be modified by multiple kinases in a stereotyped sequence: MARK initiating the early phosphorylation events followed by the action of GSK-3 β and CDK5 to modify other sites⁸. Also implicated are many other kinases including protein kinase C (PKC), cAMP-dependent protein kinase (PKA), calmodulin-dependent protein kinase (CaMKII), and mitogen-activated protein kinase ERK^{5, 9, 10}. Conversely, those kinases are capable of phosphorylating numerous substrates other than tau in the AD brain¹¹. Because of technical challenges in the analysis of the phosphoproteome, it is likely that many phosphorylation events in the development of Alzheimer's disease have not been identified thus far.

The advances in proteomics technologies, especially in phosphopeptide enrichment strategies and liquid chromatography-tandem mass spectrometry (LC-MS/MS)^{12, 13}, provide opportunities for systematic investigation of protein phosphorylation. Available strategies include immobilized metal-affinity chromatography (IMAC) enrichment incorporating metal oxides such as Fe³⁺ ion^{14, 15}, TiO₂^{16, 17}, ZrO₂¹⁸, cation and anion exchange chromatography^{19–21}, antibody capture^{22–24}, chemical derivation^{25, 26}, and the combination of these approaches^{27, 28}. More recently, calcium phosphate precipitation (CPP) has been introduced as a simple alternative method to enrich phosphopeptides, in which the phosphopeptides are pulled down by the formation of an insoluble calcium phosphate deposit²⁹. By coupling phosphate precipitation with the IMAC procedure, 227 non-redundant phosphorylation sites were identified in a rice sample²⁹. Although the CPP method alone is column-free and straightforward, it is not clear whether this method without subsequent IMAC steps is sufficient to isolate phosphopeptides for direct LC-MS/MS analysis from complex protein mixtures.

Here we describe the phosphoproteome analysis of postmortem AD brain tissue using CPP enrichment directly coupled with the LC-MS/MS approach. Although the level of protein phosphorylation may be reduced by the activities of phosphatases during extended postmortem intervals³⁰, we show that in an AD brain with a postmortem interval of as long as 20 hours, significant levels of phosphorylated proteins were still detected utilizing this simple procedure. A total of 551 phosphopeptides (466 phosphorylation sites) were identified in the brain tissue, including 379 on serine and 87 on threonine residues. To our knowledge, this is the largest scale phosphoproteome analysis conducted on human AD brain tissue to date.

Materials and Methods

Sample preparation

Frozen cortical brain tissue from a clinically and pathologically diagnosed case of definite AD (78 years old, male, 20 hrs postmortem interval, stored at -80°C) was weighed (~ 1 g) and homogenized in the lysis buffer (10 ml per gram, 20 mM Tris pH 6.8, 100 mM NaCl, 2% SDS, 1 mM EDTA, 1mM DTT, 5% glycerol, protease inhibitors and phosphatase inhibitors). The lysate was incubated at 65°C for 10 min, and centrifuged at 14,000 rpm in an Eppendorf 5417C centrifuge for 10 min. The protein concentration (~ 10 mg/ml) was estimated from the Coomassie-staining intensity on a SDS gel using titrated BSA as a standard. In order to increase the accuracy of the quantification, we run the gel for less than 5 mm to concentrate all proteins in a narrow region. The protein sample (~ 5 mg) was resolved on a SDS gel (1.5 mm thick, 35 mm wide, 35 mm long), stained and excised into ten gel pieces for in-gel digestion (20 $\mu\text{g}/\text{ml}$

trypsin, ~1:100 enzyme/substrate ratio)³¹. To prevent proteins from precipitation in the gel, the SDS concentration was adjusted from 0.1% to 0.2% in the gel and the running buffer.

Immobilized metal-affinity chromatography

The standard protocol from the company was followed to enrich phosphopeptides using PHOS-Select resin (Sigma-Aldrich). The AD sample of in-gel digestion were dried and dissolved into 30% AcN and 250 mM acetic acid, incubated with the resin for 1 hr with end-over-end rotation. The resin was then washed twice with the same loading buffer, and once with water. The bound peptides were eluted by 50 mM phosphate buffer with 1% ammonium water (pH 10.0) followed by pH adjustment, desalting and LC-MS/MS analysis.

Calcium phosphate precipitation

The calcium phosphate precipitation method was modified from a previously reported protocol as follows²⁹. The peptides from trypsin digestion were desalted by a C₁₈ column (~5 µg peptide/µl resin, Sep-Pak[®] Cartridges, Waters, Milford, MA), dried under vacuum and dissolved into 10 µl of 250 mM HOAc and 30% AcN. The desalting step after in-gel digestion is optional but we used it to remove residual salt and dye. The pH was adjusted to 10.0 by adding 60 µl of a basic buffer (50 mM KH₂PO₄, 1% ammonium water, pH 10.0). Phosphopeptides were then precipitated by the addition of 2 µl of 2 M calcium chloride. The mixture was vortexed for 3 mins and centrifuged at 14,000 rpm for 3 mins in an Eppendorf 5417C centrifuge. The pellet was washed with 50 µl of 80 mM CaCl₂, spun again to collect the pellet that was then dissolved in 20 µl of 0.1% trifluoroacetic acid (TFA) with 5% formic acid. The resulting phosphopeptides were desalted with a C₁₈ column (packed with 0.6 µl resin). The eluates from that C₁₈ desalting step were dried under vacuum and dissolved in 10 µl of 6% HOAc and 0.01% TFA for LC-MS/MS analyses.

Peptide analysis by the LC-MS/MS approach

The purified peptides were analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)³². Briefly, the peptide mixtures were loaded onto a C₁₈ column (100 µm i.d., 10 cm long, 5 µm resin from Michrom Biore-sources, Auburn, CA) and eluted during a 5–30% gradient (Buffer A: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 5% AcN; Buffer B: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 95% AcN). The eluates were monitored in a MS survey scan followed by ten data-dependent MS/MS scans on an LTQ-Orbitrap ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The Orbitrap was used to collect MS scans (350–1500 *m/z*, 1,000,000 AGC target, 1,000 ms maximum ion time, resolution 30,000), and the LTQ was used to acquire spectra of MS/MS (3 *m/z* isolation width, 35% collision energy, 5,000 AGC target, 200 ms maximum ion time).

Protein identification

The acquired MS/MS spectra were searched against the human reference database of the National Center for Biotechnology Information using the Sequest-Sorcerer algorithm (Sage-N-Research, San Jose, CA)³³, with the following parameters: semi-tryptic restriction, parent ion mass tolerance (50 ppm), oxidized methionine (+15.9949 Da) and differential modifications on serine/threonine/tyrosine (+79.9663 Da). The peptides were classified by charge state and trypticity, and then filtered by mass accuracy (15 ppm) and matching scores (XCorr). When fitted into Gaussian distribution, the standard deviation of delta-mass distribution was ~4 ppm (data not shown). XCorr is the cross-correlation score between experimental and theoretical spectra, and ΔCn is the normalized difference of XCorr scores between the 1st and 2nd peptide matches. The minimal XCorr was 1.5 for fully tryptic peptides and 2.0 for partially tryptic peptides. As ΔCn is usually small for phosphopeptides with ambiguously assigned modification sites, no ΔCn cutoff was used. All accepted proteins

sharing peptides were grouped together, in which only the top protein with highest spectral counts was selected to represent the group. The MS/MS spectra of matched phosphopeptides were further manually validated. Spectra without signature phosphate neutral losses (-49 for doubly charged, -32.7 for triply charged, and -24.5 for quadruply charged) were removed. The intensity of the neutral loss peak usually was the strongest peak in MS/MS spectra. If the strongest peak was generated by water loss or proline-directed breakage, the neutral loss peaks needed to show at least 20% relative intensity in the spectra. In some cases, the product ions on MS/MS spectra did not provide sufficient information to distinguish multiple potential phosphorylation sites in the peptides. Thus, when the two top matches of a spectrum were both phosphopeptides, and the ΔC_n was <0.1 , we considered the phosphorylation site(s) were ambiguously located in the sequence (see supplemental Table S1).

Results and Discussion

The procedure of the AD phosphoproteome analysis includes several major steps as outlined in Figure 1. Proteins from postmortem cortex were separated by 1D SDS polyacrylamide gel electrophoresis and divided into ten gel pieces, followed by trypsin digestion. Phosphopeptides were enriched by the method of calcium phosphate precipitation (CPP) and identified by the LC-MS/MS analysis. Approximately 120,000 MS/MS spectra were collected and searched against a composite target-decoy human database, rendering the identification of $\sim 6,450$ peptides. Among those, 551 peptides ($\sim 8.6\%$) are modified by phosphate group on 466 unique phosphorylation sites, which were validated by the presence of characteristic neutral loss ions in the matched MS/MS spectra.

Enrichment of phosphopeptides from AD brain tissue

Although cryopreserved AD brain tissue has been extensively used for proteomic analyses^{34–38}, global profiling of protein phosphorylation in human brain has not been reported. In addition to analytical issues of phosphoproteome studies, another major concern is that the activities of protein kinases and phosphatases are responsive to the dramatic change of cellular environment during postmortem interval, such as hypoxia, acid-base imbalance and electrolyte depletion³⁷. Whereas phosphorylation regulates myriad proteins in cellular processes, targeted proteins are transiently modified and the steady level of phosphorylated species is not abundant due to, at least partially, dominant activities of phosphatases in cells. Especially during the postmortem interval, the action of phosphatases may further diminish the global level of phosphorylation³⁰. For example, tau can be rapidly dephosphorylated by phosphatases 2A and 2B in autopsy-derived human brains³⁹. To investigate whether the residual phosphorylation events in postmortem samples can still be detected by current methodologies, we selected an AD brain specimen with a long postmortem interval (20 hrs) as the starting material.

In order to determine a phosphopeptide-enrichment method for analyzing the AD sample, we first tested the commonly used IMAC strategy (Fe^{3+} ion) in a pilot study and identified 56 phosphopeptides in a following LC-MS/MS analysis (Figure 2). In parallel, we examined a newly developed CPP method for isolating phosphorylated peptides from an identical aliquot. Instead of further purifying the coprecipitated peptides by an additional IMAC step as previously reported²⁹, we directly analyzed the enriched sample by the LC-MS/MS approach. Surprisingly, a large number ($n = 69$) of phosphopeptides were identified in the sample (Figure 2), suggesting substantial enrichment of phosphopeptides by this single step, especially considering that the analysis of the AD sample prior to the CPP step revealed no phosphopeptides (data not shown). The elution profiles of neutral loss peaks (49 u) under the IMAC and CPP conditions were compared in Figure 2A. Whereas some peptides were detected with almost equal intensity in both methods (e.g. the peak with retention time of 29.0 min),

some peptides ions were preferentially enriched in only one approach (e.g. an ion peak with retention time of 22.6 min found in the CPP sample but not in the IMAC sample). The percentage of overlapped peptides from the two methods was approximately 50% (Figure 2B). Of the phosphopeptides isolated by IMAC, ~57% carried a single phosphate group. In contrast, ~77% of the phosphopeptides enriched by CPP were mono-phosphorylated, indicating of less bias toward multiple phosphorylated peptides (Figure 2C).

In addition, the reproducibility of the CPP method was investigated by comparing two technical replicates (Figure 3). The elution profiles of the two replicates were highly similar, with respect to major ion peaks as well as putative phosphopeptides that are indicated by neutral loss of phosphate group. To further optimize the CPP condition, we sought to examine the effect of different temperatures (24°C and 4°C) and AcN concentrations (20% and 40%). However, the yield of phosphopeptides and the proportion of unmodified peptides appeared to be not affected by the testing conditions (data not shown).

Taken together, the CPP method resulted in comparable amount of phosphopeptides to the IMAC approach in this testing analysis, although the bulk of the identified peptides (~90%) by CPP was not modified but coprecipitated with calcium phosphate. The preliminary comparison of the two methods may not be viewed as a conclusive statement, as the methods might not be sufficiently optimized and the analysis was not extensively replicated. The detailed mechanisms for the difference between IMAC and CPP are not clear, and need further investigation. Nevertheless, owing to the simplicity of this single-step, column-free CPP method, all AD fractions were subsequently processed by this approach.

Identification and validation of phosphopeptides in the AD brain

The LC-MS/MS analysis of all ten gel pieces resulted in over 120,000 MS/MS spectra, approximately 18% (22,159 spectra, 6,450 unique peptides) of which were successfully matched to human peptides during Sequest database search³³. To reduce false discoveries caused by random matching or spectra of poor quality, we employed a target-decoy strategy^{40, 41} to evaluate the false discovery rate (FDR). Peptide matches were grouped according to trypticity (fully and partial) as well as precursor ion-charge state (1+, 2+, 3+, and 4+), and subsequently filtered by mass accuracy (15 ppm) and XCorr values to reduce the false discovery rate to near zero. Instead of using static XCorr cutoff, the XCorr threshold was dynamically increased to discard all peptide matches from the decoy database in every group of peptides. A total of 783 phosphopeptides passed the filtering step. As the predicted FDR has significant theoretical errors when the number of decoy matches is low⁴⁰, it is still possible that some false matches escaped this initial filtering process.

We next validated the phosphopeptide matches by removing all phosphoserine- or phosphothreonine-containing peptides without obvious precursor neutral loss of H₃PO₄ in the spectra, since the neutral loss is a well-known feature for those peptides, but is not always applicable to phosphotyrosine-containing peptides⁴². For instance, neutral loss of H₃PO₄ was observed in precursor ions and often in product ions as well (Figure 4). In contrast, a low-confidence identification of a phosphopeptide is illustrated in Figure 4A. Although the majority of *b* and *y* ions appeared to be assigned, the peak from neutral loss of H₃PO₄ is not clearly visible, and a close examination showed a discontinuous pattern of the assigned *b* and *y* ion series. The MS/MS quality was also manually examined based on the documented criteria⁴³. Finally, 551 phosphopeptides were accepted with 466 unique phosphorylation sites (81% on serine, 19% on threonine and none on tyrosine, in the supplemental Table S1 including hyperlinks to all spectra). The frequency ratio among phosphorylated Ser/Thr/Tyr is consistent with the reported⁴⁴ relative abundance, 90:10:0.05. Of those, 71% peptides are modified by a single phosphate group, and 29% are multiply phosphorylated, consistent with our testing analysis (Figure 2C). More importantly, the majority (~70%) of our dataset is not included in the

Phospho. ELM database, a resource of eukaryotic phosphoproteins from literature and high-throughput results⁴⁵.

Pathological relevance of the phosphoproteomic analysis

To characterize the identified phosphorylation sites in this study, we selected and classified the most frequently identified 20 proteins (as indicated by spectral counts) according to the cellular function (Table 1). The proteins were divided into only four groups, including neurofilaments (NFs), microtubule-associated proteins, synaptic components, and chaperones. Because synaptic failure is central to the progression of Alzheimer's disease⁴⁶, it is interesting to identify a number of key synaptic components in the sample, as exemplified by syntax-in 1 and amphiphysin II. However, it is not known if the substrates are preferentially modified in the AD. Further comparison with brain samples from healthy individuals and other disease controls (e.g. Parkinsonism) is needed to address this issue.

As expected, microtubule-associated protein tau was found to be phosphorylated at four residues (Ser46, Thr50, Thr181 and Ser404). Both Ser46, Thr 181 and Ser404 have been shown to be modified in the tau aggregates, and all four sites can be targeted by GSK-3 β *in vitro*⁴⁷. Abnormal tau phosphorylation has been consistently observed in AD brains and is thought to be linked to pathogenesis. Eliminating all SP/TP phosphorylation sites of tau dramatically reduces the toxicity as shown by fly eye morphogenesis⁴⁸, and pseudo-phosphorylated tau elevates the toxicity in a cellular model⁴⁹. Moreover, pathological tau presumably sequesters other microtubule-associated proteins (MAPs) and therefore disassembles microtubules⁵. Intriguingly, MAP1A, MAP1B and MAP2 are extensively modified by phosphorylation in the AD sample (Table 1). For example, a total of 53 MAP1B phosphorylation sites (194 spectral counts) emerged from this analysis. Like tau, the hyperphosphorylation of other MAPs destabilizes the microtubule network and might also play roles in the development of neurodegenerative disease. Indeed, knockout of the MAP1B homologue in *Drosophila* causes defects in the neuronal cytoskeleton and progressive neurodegeneration⁵⁰. The involvement of MAP1B hyperphosphorylation in neurodegeneration is not well understood and merits future investigation.

Conclusion

We present here our initial findings representing the first large-scale phosphoproteome analysis of an AD postmortem brain. The feasibility of extracting and recovering phosphopeptides from autopsy brain specimens is demonstrated. The simple approach of coupling calcium phosphate precipitation with LC-MS/MS exhibits a reasonable coverage of phosphorylation sites. Future studies will focus on quantitative phosphoproteome analysis comparing AD and control brains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Flowchart of the experiment to analyze the phosphoproteome in the AD brain.

Figure 2.

Comparison of IMAC and CPP methods. A. Two identical samples were processed by both methods coupled with LC-MS/MS. The LC elution profiles of neutral loss (49 Da) were shown for comparison. The two inserts indicate the MS intensity of two representative peptide ions with neutral loss. B. The overlap of phosphopeptides identified in both methods. C. The distribution of peptides with respect to the number of phosphorylation sites.

Figure 3.

Reproducibility of the calcium phosphate precipitation approach. A brain protein sample was equally split into two technical replicates and run on a short SDS gel (5 mm). The proteins in the entire gel lane were digested followed by the CPP step and LC-MS/MS. Indicated are the elution profiles of the replicates and the neutral loss plots of phosphate group for doubly charged peptides.

Figure 4.

Representative CID MS/MS spectra of incorrectly/correctly assigned phosphopeptides. A. One MS/MS spectrum of a Sequest-derived phosphopeptide was discarded during manual validation due to the lack of neutral loss. B. A typical spectrum of a phosphopeptide in the AD sample displayed dominant precursor neutral loss peak. C. A phosphopeptide spectrum in which the neutral loss ion is not the most abundant product ion because proline breakage is the main event during the fragmentation of the peptide. D. Another phosphopeptide spectrum shows the prevalence of neutral loss in product ions.

Table 1
The list of abundant phosphorylated proteins detected in the AD brain

Protein names	GenBank [™] accession number	# spectra of phosphopeptides	# phosphorylation sites
Neurofilaments			
neurofilament 3 (150kDa medium)	NP_001099011.1	591	18
neurofilament, heavy polypeptide 200kDa	NP_066554.2	59	27
Microtubule-associated proteins			
microtubule-associated protein 1B	NP_005900.2	194	53
microtubule-associated protein 1A	NP_002364.5	46	18
microtubule-associated protein 2	NP_002365.3	27	5
microtubule-associated protein tau	NP_005901.2	14	4
Other cytoskeletal proteins			
adducin 1 (alpha) isoform b	NP_054909.2	125	4
adducin 2 isoform c	NP_059517.1	29	12
spectrin, beta, non-erythrocytic 1	NP_003119.2	30	10
ankyrin 2, isoform 1	NP_001139.3	24	9
ankyrin 2, isoform 2	NP_066187.2	21	7
Synaptic components			
syntaxin 1A	NP_004594.1	27	2
syntaxin 1B	NP_443106.1	29	2
amphiphysin II	NP_647597.1	43	3
GAP-43	NP_002036.1	23	9
NAP-22/BASP1	NP_006308.3	63	4
A-kinase anchor protein 12 (AKAP12)	NP_005091.2	37	9
myristoylated alanine-rich PKC substrate (MARCKS)	NP_002347.4	21	2
neural cell adhesion molecule 1 isoform 2 (NCAM1)	NP_851996.2	16	2
Chaperones			
Hsp90	NP_031381.2	52	3