

LC/MS/MS based strategies to identify novel phosphorylation sites within proteins implicated in neurological diseases

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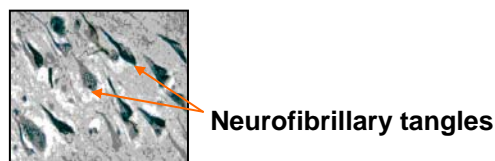
Introduction

We have used LC/MS/MS to discover novel sites of phosphorylation within key proteins implicated in the progression of neurological diseases, for example Alzheimer's disease (AD).

A pathological feature of AD is the accumulation in brain neurons of abnormally hyperphosphorylated tau. This results in its dissociation from micro-tubules and polymerisation into tangles of paired helical filaments (PHF) as shown (Figure 1). Such aggregates are responsible for neuronal cell death, a central feature of AD

The prevention of tangle formation is therefore an important therapeutic goal towards the treatment of AD. Here we describe insights gained into the phosphorylation of PHF tau and the kinases involved. In addition subtleties in the MS analysis of phosphopeptides are discussed with particular emphasis on the interpretation of MS/MS spectra for the correct assignment of phosphorylation.

Figure 1 - Light microscopy of AD brain



Methods

Characterisation of protein phosphorylation is typically undertaken using purified phosphoproteins separated using 1D SDS-PAGE. Colloidal coomassie stained bands are digested with a combination of proteases (trypsin, chymotrypsin, Asp-N) to maximise MS/MS sequence coverage. Chromatographic separations were performed using an Ultimate LC system (Dionex, UK) and a Q-ToF *micro* (Waters, UK) for MS/MS analysis. Database searching was performed using the Mascot algorithm (Matrix Science, UK). All MS/MS spectra relating to phospho-peptides were then subsequently visually verified to confirm the phosphorylation site(s) indicated.

Results and Discussion

Part 1 - Summary of PHF tau phosphorylation

The polypeptide sequence of tau illustrating sequence coverage and phosphorylation sites observed during the LC/MS/MS analysis of enzymatic digests of PHF-tau is shown to summarise the results of these experiments (Figure 2). Identification of 12 new phosphorylation sites has led to further studies to identify the specific kinases involved in the phosphorylation of PHF tau. We have characterised the phosphorylation sites of recombinant tau phosphorylated by known selected kinases and also a rat brain cell lysate, which contains a pool of cellular kinases. This has allowed us to closely emulate PHF tau phosphorylation. This system serves as a good model of protein phosphorylation and has provided us with information-rich datasets from which we have gained important insights into the analysis of phosphopeptides and tau biology.

Figure 2 – Sequence of tau protein illustrating coverage and phosphorylation sites observed

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1 MAEPRQEFV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT
51 PTEDEGSEEPG SETSDAKSTP TAEDVTAPLV DEGAPGQAA AQPHTEIPEEG
101 TTAEBAGIGD TPSLEDEAAG HVTQARMVSK SKDGTGSDDK KAKGADGKTK
151 IATPRGAAPP GQKQANATR IPAKTPPAK TPPSSGSEPK SGDRSGYSSP
201 GSPGTPGSRS RTPSLPTPPT REPKVAVVR TPPKSPSSAK SRLQTAPVPM
251 PDLKNVSKI GSTENLKHQP GGGKVQIINK KLDLSNVQSK CGSKDNIKHV
301 PGGGSVQIVY KPVDLSKVTS KCGSLGNIH KPGGGQVEVK SEKLDDFKDRV
351 QSKIGSLDNI THVPGGNKK IETHKLTFRE NAKAKTDRGA EIVYKSPVVS
401 GDTSPRHLN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L
    
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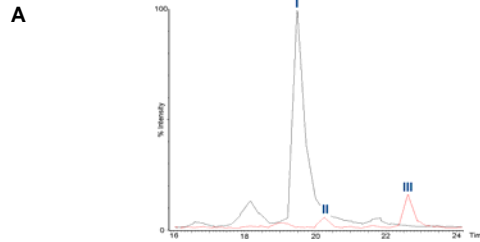
Key:

■ 91.4% sequence coverage of full length Tau based on recent LC/MS/MS data.

■ Phosphorylated residues identified by LC/MS/MS (also found previously by MS/MS and Edman sequencing).

12 novel phosphorylation sites including isoform specific sites. These are not indicated due to ongoing patent application process.

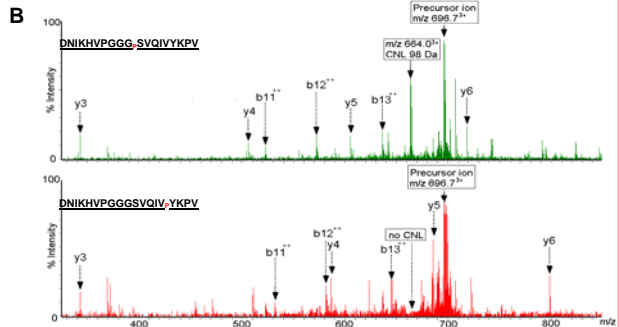
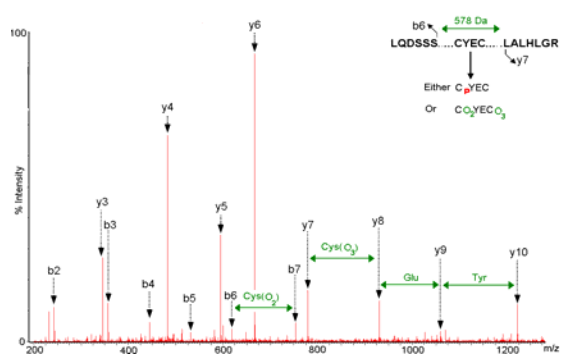
Figure 3 – Chromatographic resolution of two regiomeric forms of a phosphopeptide



Panel A LC/MS chromatogram showing differences in retention times (RT) and intensities (BPI) of :

- I** DNIKHVPGGGSVQIVYKPV (m/z 669.7³⁺, RT 19.6min, BPI 4000)
- II** DNIKHVPGGGSVQIVYKPV (m/z 696.7³⁺, RT 20.3min, BPI 230)
- III** DNIKHVPGGGSVQIVYKPV (m/z 696.7³⁺, RT 22.7min, BPI 680)

Figure 4 - The MS/MS spectra of peptide LQDSSSCYECLALHLGR (m/z 988.42²⁺)



Panel B MS/MS spectra of both phosphopeptides

The 'b¹¹⁺' and 'y' ions are highlighted, which confirm phosphorylation at serine 11 (upper spectrum) and tyrosine 16 (lower spectrum).

No CNL is observed in the lower spectrum. This is characteristic of tyrosine phosphorylation.

Part 2 - Insights into MS analysis of phosphopeptides

Phosphopeptides with the same m/z but different site of phosphorylation (regiomers) are sometimes chromatographically resolved as shown in the example (Figure 3). Co-elution of regiomers can also be observed in these datasets. In such cases the MS/MS spectra contains ions relating to a mixture of phosphorylated forms. Mascot only lists the single most likely sequence, therefore to fully appreciate the heterogeneous nature of these spectra visual verification is paramount.

Part 3 - The importance of visual verification

The MS/MS spectra of LQDSSSCYECLALHLGR (m/z 988.42²⁺) is shown (Figure 4). *De novo* sequencing of this peptide, revealed that unexpected oxidation of cysteine residues had occurred. Mascot indicated a possible pTyr but the peptide actually contains two cysteines in different oxidation states. We conclude that other alternatives need to be considered where phosphorylation assignment is ambiguous.

Conclusions

The roles of specific kinases present in rat brain cell lysate are currently being explored to attribute their importance in PHF tau phosphorylation by assessing the changes in tau phosphorylation brought about by inhibiting these key tau kinases. This has led to the identification of new targets for the therapeutic intervention of AD.