

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

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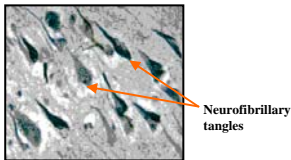
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INTRODUCTION

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 microtubules and polymerisation into tangles of paired helical filaments (PHF). Such aggregates are responsible for neuronal cell death, a central feature of AD.

Figure 1 Light microscopy of AD brain



The prevention of tangle formation is therefore an important therapeutic goal towards the treatment of AD.

This poster describes insights gained into the phosphorylation of PHF tau and the kinases involved. In addition subtleties in the MS analysis of phosphopeptides will be discussed with particular emphasis on the interpretation of MS/MS spectra for the correct assignment of phosphorylation.

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.

METHODS

Chromatographic separations are performed using an Ultimate LC system (Dionex, UK) and a Q-ToF *micro* (Waters, UK) for MS/MS analysis¹. Database searching is performed using the Mascot algorithm (Matrix Science, UK). All MS/MS spectra relating to phosphopeptides are then subsequently visually verified to confirm the phosphorylation site indicated.

RESULTS

Summary of PHF tau phosphorylation

Figure 2 Full length sequence of tau

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1 MAEPFQEFV MEDHAGTYGL GDRKDGQGY MHQDQGDIT AGLKESPIQT
51 PTEDESEFG SPTSDAKSTP TAEVDAPLV DEGAPGQAA AQPHTIPEG
101 TFAEAGIGD TPSLEDEAAG HVTQARMVSK SKDGTGSDK KAKGADGKTK
151 IATFRGAAPP GQKQGANATR IPAKTPPAK TPFSGEPFK SGRSGVSSP
201 GSPGTPGRRS RTPLETFPT REPRVAIVK TPFGSPSAK SRLQTAVPM
251 PDLNYSKSI GFENLEKHP GSKKQILNK KDLNYSKSI GSSNMLKIV
301 PGGSGVQIVY KPVLSKVTI KCSLSLNHH KPGGQVEVK SEKLDKDRV
351 QSKIGSLNDI THVPGGNKK IETHKLTPE NAKAKTDGA EIVYKSPVVS
401 GDTSPHLSN VSGTSGIDMV DSPQLATLAD EVSASLAKQ L
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91% sequence coverage obtained by a combination of proteolytic enzymes and LC/MS/MS.

26 known sites of serine and threonine phosphorylation identified by either LC/MS/MS, nano-electrospray, Edman sequencing or antibodies².

12 novel sites of phosphorylation identified by LC/MS/MS (novel sites not indicated).

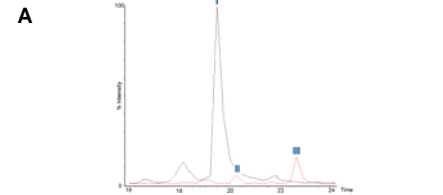
Identification of these new sites has led to further studies to identify the specific kinases involved in the phosphorylation of PHF tau. We have characterised the phosphorylation sites of recombinant tau phosphorylated by known selected kinases and also a rat brain cell lysate, which contains a pool 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.

RESULTS

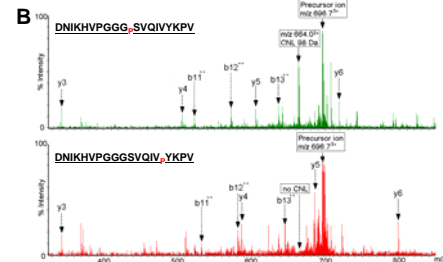
Insights into MS analysis of phosphopeptides

Figure 3 LC/MS/MS analysis of recombinant tau phosphorylated *in vitro* with rat brain cell lysate



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)



Panel B MS/MS spectra of both phosphopeptides

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

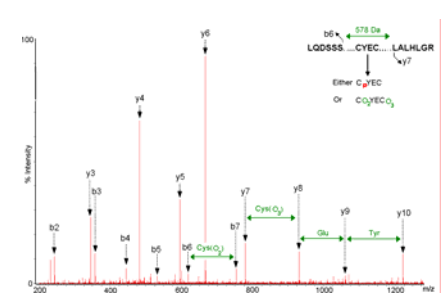
No CNL is observed in the lower spectrum. This is characteristic of tyrosine phosphorylation.

In this example phosphopeptides with the same m/z but different site of phosphorylation (regiomers) are chromatographically resolved. Co-elution of regiomers can also be observed in these datasets. Here 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.

RESULTS

The importance of visual verification

Figure 4 MS/MS spectra of LODSSSCYECLALHLGR (m/z 988.42²⁺)



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.

DISCUSSION

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.

REFERENCES

- Standen CL, et al. (2003) The neuronal adaptor protein FE65 is phosphorylated by ERK1/2. *Mol Cell Neurosci.* **24** 851-857
- Hanger D, et al. (1998) New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nano-electrospray mass spectrometry. *J.Neurochem.* **71** 2465-76