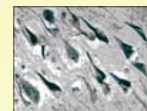


Introduction

A pathological feature of Alzheimer's disease (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 in the light microscopy image below (Figure 1).

Figure 1 Light microscopy of AD brain



Neurofibrillary tangles

Such aggregates are responsible for neuronal cell death, a central feature of AD. Currently there are two tau-related biomarkers that have been evaluated in human CSF, namely total tau (T-tau) and phospho-tau (P-tau).

More recently CSF P-tau assessments have focused on different phospho-peptides but these are limited to a few sites within the protein.

The determination of the phosphorylation sites on PHF tau has been undertaken using mass spectrometry and many new sites have been discovered (Hanger *et al*, 2007). Antibodies to detect many of these sites do not currently exist and it is both a challenging and lengthy process to produce reagents with the appropriate selectivity and specificity. Recent developments in mass spectrometry now enable the screening of tau phosphorylation sites independent of disease status, species of origin, or location within the protein. We present results to demonstrate the application of mass spectrometry to the analysis of tau phosphorylation in clinical and pre-clinical samples. It is envisaged that such methods will provide the basis for the most comprehensive phospho-site specific array for tau available within the AD research community as well as having applicability to other neurological disorders.

Methods

Tau was enriched by biochemical techniques using protocols we have previously established and demonstrated for both human brain samples as well as material for transgenic mice. Gel electrophoresis is used as the final step in this process. Tau related bands are then excised from the gel and an enzymatic digest is performed to produce a mixture of peptides and phospho-peptides. A panel of enzymes is recommended to extend the sequence coverage of the protein and, in some instances, provide an overlapping set of phospho-peptides. Each digested sample is then analysed using a combination of mass spectrometry strategies as described. All chromatographic separations are performed using an Ultimate LC system (Dionex, UK) and initial MS/MS data is acquired using a Q-ToF *micro* (Waters, UK). Database searching is performed using the Mascot algorithm (Matrix Science, UK). All MS/MS spectra relating to phospho-peptides are then subsequently visually verified to confirm the phosphorylation site indicated. Alternatively a dedicated precursor m/z 79 can be used to enhance the detection of low abundant phosphorylated species (see Figure 2). Here the mass spectrometer is set to record all molecules that produce the fragment ion of m/z 79, being PO₃⁻, which is produced when phosphorylated molecules are subjected to MS/MS.

Discovery Experiments

The LC/MS/MS analysis of PHF-tau revealed many sites of phosphorylation on serine and threonine residues including 12 novel sites. Additionally phosphorylation of Tyr394 was also shown to be present (Derkinderen *et al*, 2005).

Figure 3 Phosphorylation sites on PHF-tau from AD brain



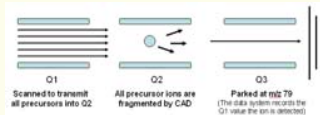
Identification of these new sites has led to further studies to identify the specific kinases involved in the phosphorylation of PHF tau. We have characterised the phosphorylation sites of recombinant tau phosphorylated by known selected kinases and also a rat brain cell lysate, which contains a pool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

Importantly, these experiments indicate that casein kinase 1 delta (CK-1δ) can phosphorylate tau at additional sites to those modified by the more established tau kinases such as GSK3β and CDK5. We also demonstrate that c-Abl is the kinase responsible for in vivo phosphorylation of Tyr 394 in PHF-tau.

Summary of Discovery Phase

- Several novel sites have been discovered which are selectively phosphorylated by casein kinase 1 delta (CK-1δ)
- One site has been discovered at Tyr 394 which is selectively phosphorylated by the kinase c-Abl
- CK1 and c-Abl represent potential new targets for AD therapy and have biomarker potential

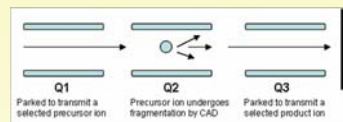
Figure 2 Schematic to illustrate precursor ion m/z 79 scan



Development of Portable assays

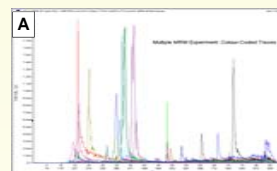
Multiple reaction monitoring (MRM) mass spectrometry has been used for many years as a diagnostic and forensic tool to accurately quantify the levels of toxins and drug metabolites. MRM is the scan type with the highest duty cycle and is used for monitoring one or more specific ion transition(s) at high sensitivity. Here, Q1 is set on the specific parent m/z (Q1 is not scanning), the collision energy is set to produce the optimal diagnostic charged fragment of that parent ion, and Q3 is set to the specific m/z of that fragment. Only ions with this exact transition will be detected. A schematic is shown to illustrate the mechanics of an MRM experiment (Figure 4).

Figure 4 Schematic to illustrate an MRM scan



The feasibility of using MRM as the basis of site directed tau phosphorylation assays has been demonstrated and is illustrated below (see Figure 5). These assays are effectively independent of sample type or species of origin hence the quantitative measurement of tau phosphopeptides by mass spectrometry represents a powerful alternative to antibodies. The area under the MRM LC peak is used to quantitate the amount of the analyte present. In a typical quantitation experiment, a standard concentration curve is generated using a synthetic version of the phospho-peptide of interest. When the unknown sample is then run under identical conditions, the concentration for the peptide in the unknown sample can be determined using the peak area and the standard concentration curve.

Figure 5 MRM assays for tau phosphorylation



Relevant Sample:

- Recombinant protein
- Cell lines
- Mouse models
- PHF tau (Human)

Creation of panels:

- Choice of protease
- Site directed analysis

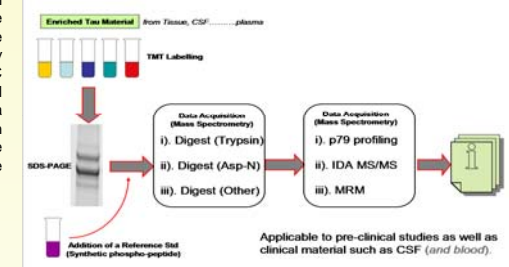
Panel A shows the total ion chromatogram (TIC) generated for 41 MRM transitions designed to selectively monitor 15 phosphorylation sites within tau protein. In this example PHF-tau from post mortem human brain was purified and enzymatically digested using trypsin. Assay criteria can be optimised for particular sites and the inclusion of synthetic phosphopeptides enables the absolute concentrations of each individual phosphopeptide to be established.

TMT Assays for Extra Multiplexing

The use of isobaric reagents, such as Proteome Sciences proprietary Tandem Mass Tags (TMT[®]) molecules, which enable quantitation at the MS/MS level rather than MS, are likely to extend the ability to multiplex samples. Ultimately this will allow higher throughput analysis and provide a significant advantage in terms of speed and cost of the experiments. A combination of TMT[®] and MRM may offer further advantages and experiments are currently in progress to evaluate this particular strategy.

A schematic to illustrate the overall concept of tau phosphorylation assays by mass spectrometry is shown (Figure 6). Here, quantitative measurements are enabled via the use of Tandem Mass Tag (TMT) reagents, Proteome Sciences proprietary labels. In the workflow depicted TMT sixplex reagents are used to label five individual tau protein preparations. These are mixed prior to SDS-PAGE and the tau band is excised ready for enzymatic digestion. A synthetic TMT-labelled standard phospho-peptide is added at this stage. Relative and absolute quantitation of individual tau phospho-peptides is achieved by comparing the intensity of the TMT reporter ions created during MS/MS

Figure 6 Schematic depicting a multi-site phospho-assay system



Conclusions

- MS workflows (p79, MRM) have been established and are available to screen and quantify phosphorylation sites in tau and other proteins
- TMT[®] enhances the ability to multiplex larger numbers of samples.

References

Hanger *et al* (2007) Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *J Biol Chem.* 282(32):23645-54.
 Derkinderen *et al* (2005) Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase. *J Neurosci.* 25(28):6584-93.