Development of Sensitive Proteomic Approaches for Protein Tyrosine Phosphorylation Detection

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as a combination of conventional narrative and portfolio of publications by

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Abstract

The elucidation of the complex array of cell signalling cascades is imperative for a deeper understanding of cell biology in both physiological and patho-physiological states. Extensive biochemical characterisation of signalling networks has revealed the importance of post-translational modifications (PTMs), particularly phosphorylation. Signalling via protein phosphorylation occurs across homeostatic proliferative, differentiative and anti-apoptotic events. Dysregulation of the kinase signalling pathways as well as mutations in kinases involved in phosphorylation have been implicated in a number of pathologies such as cancer or immune deficiencies. While it is estimated that 50% of all proteins are phosphorylated during their lifetime, phosphorylated proteins are present in relatively low abundance compared to their non-phosphorylated counterparts. The rarity of phosphorylation, which occurs on serine, threonine and tyrosine residues, has prompted the development of sensitive approaches to improve phosphorylation characterisation. Proteomic-based strategies offer novel approaches to overcome the limitations of currently available strategies for phosphoprotein analysis. The research presented within describes the development of proteomic-based methodologies for phosphotyrosine identification, quantitation and characterisation. These methods utilise the anti-phosphotyrosine Antibody 4G10 along with other MS-compatible approaches for phosphotyrosine enrichment prior to MS analysis. Methods for more targeted phosphoprotein analyses involved coupling of 4G10 covalently to super para-magnetic beads or by affinity to super para-magnetic beads with protein G covalently attached. These 4G10-coupled beads successfully enriched tyrosine phosphopeptides derived from tryptic phosphoprotein digests for identification and characterisation of phosphopeptides using MALDI-TOF/TOF MS analysis.

The limited capacity of the magnetic bead approach for analysis of more complex samples necessetated the development of a more global proteomic strategy for tyrosine phosphorylation analysis. A global strategy that provides not only qualitative pTyr information but also shows quantitative changes that occur with pTyr signalling is imperative for detailed signalling cascade analyses. The global approach presented here utilised the 4G10 Ab/bead approach as well as Hydrophilic interaction chromatography (HILIC) for the enrichment of pTyr peptides from complex samples isotopically-labelled to quantify tyrosine phosphorylation after LC-MALDI-TOF/TOF MS analysis. Aspects of this approach were modified to improve phosphopeptide detection and characterisation, including the development of a novel optimised matrix-deposition strategy for LC-MALDI-TOF/TOF MS. The strategy, termed EZYprep LC, allowed the effective use of the atypical 2,5-

DHB matrix with phosphoric acid to improve phosphopeptide ionisation and subsequently identify and characterise more phosphorylation sites on phosphoprotein samples compared with LC-ESI-IT-MS/MS.

Another aspect of the global strategy was the development of a modified isotope protein coded label strategy (modified ICPL). The optimised ICPL approach ensured quantitative information from a larger sub-set of peptides after tryptic digest of complex samples. The improved ability to quantify using this approach was highlighted by a comparative analysis of complex cell lysates labelled using the conventional ICPL strategy and the modified ICPL strategy. The modified ICPL labelling strategy identified more proteins and provided more quantitative information that the conventional ICPL methodology. As such, the global phospho-tyrosine strategy, combined the modified ICPL labelling and 4G10 Ab/bead enrichment with peptide fractionation and MALDI-TOF/TOF MS analysis, was subsequently utilised to identify and quantify tyrosine phosphorylation occurring in insulin-stimulated insulin receptor A- and B-subtypes.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Mark Condina and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (as listed in the publications list below) resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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"The larger the island of knowledge, the longer the shoreline of mystery." *Unknown author*

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Publications

Within Thesis:

Condina, M. R., Guthridge, M. A., McColl, S. R., Hoffmann, P., A sensitive magnetic bead method for the detection and identification of tyrosine phosphorylation in proteins by MALDI-TOF/TOF MS. *Proteomics* 2009, *9*, 3047-3057 – **Chapter 2**

Condina, M. R., Gustafsson, J. O. R., Klingler-Hoffmann, M., Bagley, C. J., McColl, S. R., Hoffmann, P., EZYprep LC-coupled MALDI-TOF/TOF MS: An improved matrix spray application for phosphopeptide characterisation. *Proteomics* 2010, 10, 2516-2530 – **Chapter 3**

Arising from Thesis:

Condina, M.R., Klingler-Hoffmann, M. and Hoffmann, P. Tyrosine Phosphorylation Enrichment and Subsequent Analysis by MALDI-TOF/TOF MS/MS and LC-ESI-IT-MS/MS. *Current Protocols in Protein Science* 2010, 62:13.11.1-13.11.26 – **Appendix 7B**

Commonly-Used Abbreviations

µL	Microlitre
µ-WPS	Micro-Well-plate-sampler
1-DE	one-dimensional poly-acrylamide gel electrophoresis
2-DE	two-dimensional poly-acrylamide gel electrophoresis
Å	Angstrom
AA	Amino acid
Ab	Antibody
Abs	Antibodies
AC	Affinity Chromatography
ACN	Acetonitrile
ACQA	Absolute quantitation
BS	Binding solution
BSA	Bovine Serum Albumin
C*	Carboxamidomeythl cycteine
CHCA	α-cyano-4-hydroxycinnamic acid
CHCA SMW	CHCA sample matrix wash
CID	Collision induced dissociation
Cov-P	Covalently-coupled 4G10 MB-covAC-Select
Da	Dalton
DD	Dried Droplet
DE	Delayed extraction
DHB	2,5-dihydroxybenzoic acid
DHB ML	DHB matrix layer
DIGE	Difference Gel Electrophoresis
DTT	Dithiothreitol
e	Elementary charge
ECD	Electron capture dissociation
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
E_k	Kinetic
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
FA	Formic acid
FACS	Fluorescence activated cell sorting

Fc	Fraction Collector
fmol	Femtomole
FTICR	Fourier-transform ion cyclotron resonance
G-250	Colloidal coomassie
GFPB	Glufibronopeptide B
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAP	Hydroxyapatite enrichment
HCl	Hydrochloric acid
HE	Hydrophobic eluate fraction
HFT	Hydrophobic flow through fraction
HIC	Hydrophobic interaction Chromatography
HIE	Hydrophilic eluate fraction
HIFT	Hydrophilic flow through fraction
HILIC	Hydrophilic Interaction Chromatography
HPLC	High performance liquid chromatography
I.D.	Inner diameter
IAA/IAM	Iodoacetamide
IC_{50}	The half maximal inhibitory concentration
ICAT	Isotope coded affinity tags
ICPL	Isotope coded protein labels
IDA	Iminodiacetic acid
IEX	Ion exchange Chromatography
IGF	Insulin-like growth factor
IGFBPs	IGF-binding proteins
IMAC	Immobilised metal affinity Chromatography
IP	Immuno-precipitation
IPA	Ingenuity pathways analysis
IR	Insulin Receptor
ISD	In-source decay
IT	Ion Trap
iTRAQ	Isotope tagging for relative and absolute quantitation
L	Length
LC	Liquid Chromatography
LDS	Lithium Dodecyl Sulphate
LDS	Lithium dodedyl sulphate
LIT	Linear Ion Trap
m	Mass

M*	Oxidised methionine
m/z	Mass-to-charge
MALDI	Matrix assisted laser desorption/ionisation
MAP	Mitogen-activated protein kinase
MAPK	MAP kinase
MB-covAC-Select	Magnetic bead based covalent affinity Chromatography for binding of freely selectable proteins
MB-IAC Prot G	Magnetic bead based immunoaffinity Chromatography on immobilised protein G
MB-IMAC Fe	Magnetic bead-IMAC Fe
MCP	Microchannel plate
MOAC	Metal oxide affinity Chromatography
Mr	Relative molecular mass
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS/MS	Tandem MS
NaF	Sodium fluoride
nLC	Nano-LC
NP-40	Nonidet P-40
NTA	Nitriloacetic acid
OVA	Ovalbumin
PA	Phosphoric acid
PAGE	poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
pI	Isoelectric point
PM1	Protein mix 1 (1 pmol BSA, 1 pmol OVA, 2 pmol β-cas)
pmol	Picomole
PMSF	Phenylmethanesulfonylfluoride
Pro-GP	4G10 affinity-coupled MB-IAC Prot G
PSD	Post-source decay
pSer	Phosphoserine
PTEN	Phosphatase and tensin homolog
pThr	Phosphothreonine
PTKs	Protein tyrosine kinases
PTMs	Post-translational modifications
PTPs	Protein tyrosine phosphatases
pTyr/pY	Phosphotyrosine
PVDF	Polyvinylidene fluoride

Q	Quadrupole
QQQ	Triple quadrupole
qTOF/QTOF	Quadrupole time of flight
Rf/RF	Radio frequency
RIPA	Radioimmunoprecipitation assay buffer
RP	Reverse Phase Chromatography
R^{-ve}	IGF-1R-deficient mouse embryo fibroblast
SA	sinapinic acid
SAX	Strong anion-exchange
SCX	Strong cation-exchange
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEC	Size exclusion Chromatography
SF1	Sheath fluid containing 0.05% PA/0.05% TFA/50% ACN
SILAC	Stable isotope labelling by amino acids in cell culture
SILE	Stable isotope labelling experiment
SIMAC	Sequential elution from IMAC
SRM	Single reaction monitoring
t	Time
TBST	Tris-buffered saline Tween-20
TED	Tris(carboxymethyl)ethylenediamine
TFA	Trifluoroacetic acid
TiO_2	Titanium Dioxide
TIS	Timed ion selector
TL	Thin-layer
TLC	Thin-layer Chromatography
TOF	Time-of-Flight
U	Voltage
v	Velocity
WAX	Weak anion-exchange
WCX	Weak cation-exchange
YAG	yttrium aluminium garnet
z	Charge
β-cas	β-casein
βic	β-intracellular domain of the granulocyte-macrophage colony-stimulating factor