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Phosphoproteome of crab-eating macaque cerebral cortex characterized through multidimensional reversed-phase liquid chromatography/mass spectrometry with tandem anion/cation exchange columns

Quan Quan [a], Jinwen Feng [b], Leong-Ting Lui [a], Tieliu Shi [b], Ivan K. Chu [a,*]

[a] Department of Chemistry, The University of Hong Kong, Hong Kong, China
[b] Center for Bioinformatics and Computational Biology, and The Institute of Biomedical Sciences, School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China

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ABSTRACT

A fully automated online multidimensional liquid chromatography (MDLC) platform featuring high-/low-pH reversed-phase (RP) dimensions and two other complementary—strong anion exchange (SAX) and strong cation exchange (SCX), respectively—chromatographic separations in tandem, with conventional offline titanium dioxide pre-enrichment, has been applied for the first global phosphopeptide identification from the macaque cerebral cortex in the presence of phosphatase inhibitors. Phosphorylation data interpretation, including site determination, and network construction have been performed: 14,338 distinct phosphopeptides in 7572 non-redundant phosphosites at 1% FDR were identified with 784 *novel* phosphorylation sites when mapping into the two most-curated public phosphorylation databases, PhosphoSitePlus (PSP) and Phospho.ELM (ELM), using probability-based placements. The net charges of both extremely acidic and basic phosphopeptides depend largely on the pH of the solvent, in turn impacting their retention and subsequent fractionation; the inclusion of the complementary SAX and SCX column chemistries after the high-pH RP dimension allowed effective retention and separation of net-negatively and −positively charged phosphopeptides, thereby leading to extended anionic and cationic phosphopeptide coverage from basophilic and acidophilic kinase substrates. A valuable protein interaction network of known and predicted motifs kinases was constructed from 3064 confident phosphorylation sites in the non-human primate's brain.

1. Introduction

The brain is an extraordinarily sophisticated and multifaceted organ that controls most of the body's activities while facilitating highly specialized functions (e.g., memory, learning, cognition) [1]. The cellular organization of this organ is extremely complex, comprising a multitude of cell types intertwined and intricately organized into circuit assemblies [1]. Therefore, it is not surprising that a wide spectrum of disorders are linked to perturbations of this heterogeneous cellular network, with more than 1000 different ones being associated with brain and central nervous system dysfunction [2]. Because proteins are the main cellular functional components, comprehensive characterization of their expression, interaction, organization, and function from a global perspective—namely, "neuroproteomics"—is required to understand the complex biology of the brain [3]. One aspect of the field of neuroproteomics involves examining the proteome from a functional perspective, assessing the essential role of post-translational modifications (PTMs) and their implications on protein function. Of the various PTMs, reversible phosphorylation is the most predominantly observed, with approximately one-third of the proteome being phosphorylated at any given time [4]. Phosphorylation is a reversible process in which a phosphate group (PO_4^{3-}) is added to an amino acid side chain through a condensation reaction. Recent studies of protein phosphorylation have found that phosphorylation events occur mainly on serine, threonine, and tyrosine residues [5], although there are also reports of phosphorylation on histidine, aspartate, cysteine, lysine, and arginine residues [6–8]. Reversible phosphorylation has been implicated in nearly every cellular process, including neuron-specific ones (e.g., vesicle transport and

* Corresponding author.
E-mail address: ivankchu@hku.hk (I.K. Chu).

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exocytosis), Ca^{2+}-dependent signaling, receptor desensitization, and endocytosis, and is a central regulatory mechanism modulating protein function in the developing and mature brain [9–12]. Protein phosphorylation has a major influence on cell signaling through the reversible addition or removal of phosphate groups, regulating a diversity of protein localization, degradation, conformation, folding, and functions, such as protein activities, and substrate specificities via protein kinase, protein phosphatase, or phosphor-binding protein with predefined phosphorylation motifs [13]. Protein kinases regulate many aspects of cellular behavior, resulting in modulation of protein function or stability, changes in association with other proteins, or the movement of proteins from one cellular compartment to another [14]. The execution and modulation of this dynamic cellular signal transduction mechanism is governed by a complex coordinated interplay between over 500 kinases and 150 protein phosphatases [15]. The activities of kinases and phosphatases are themselves fine-tuned by their phosphorylation states, resulting in a complex system-wide regulatory pattern with interconnected signaling pathways [15,16]. With its central role in regulating protein function, abnormalities in protein phosphorylation have been implicated in a wide spectrum of neurodegenerative diseases, including amyotrophic lateral sclerosis and Alzheimer's and Parkinson's diseases [17]. Therefore, to shed light on the role of reversible phosphorylation *in vivo* with regard to normal neuronal cell function and associated disease conditions, extensive phosphoproteomic profiling of various neuronal tissues from animal models (e.g., mouse, rat) has been conducted [12,15,16]. Although rodent models provide important insight into underlying biology, they cannot completely reflect the human physiology and diseased states. To address this issue, the focus must shift toward the study of more phylogenetically comparable models with reduced translational barriers (e.g., non-human primates). Among non-human primate models, the brain structure, neurovasculature, and cortical anatomy of macaque monkeys (genus *Macaca*)—particularly *Macaca fascicularis*—closely resemble those of humans [18–20]. Also known as crab-eating macaques, they have become one of the most commonly used non-human primates in biomedical research, primarily in studies of neurobiology and neurophysiology and in clinical research [18,21–23]. Several neurodegenerative disease models—including aging, Alzheimer's disease, epilepsy, Parkinson's disease, and stroke—have been developed and implemented successfully using this model [22–28].

Protein phosphorylation appears to have a close relationship with multiple forms of cancer and neurodegenerative diseases, depending on the location, selectivity, nature, and type of the phosphorylation recognition domains on the kinase motifs being involved (e.g., whether tandem multiple phosphorylation or consensus sequence types exist around a given phosphorylation site) [29–31]. Protein kinases can be divided, based on their substrate specificity, into three major classes: Pro-directed, basophilic, and acidophilic motifs [32]. Acidophilic and Pro-directed sites make up a large portion in these data sets. Proteins involved in signal transduction and signaling cascades, however, exhibit a distinct distribution, with a low frequency of acidic phosphorylated sequences and higher numbers of basic sequences. Thus, the ability to efficiently identify corresponding phosphorylation sites and motifs is an important step toward revealing the underlying complex biochemical processes occurring via phosphorylation. Nevertheless, analytical challenges remain while attempting to cope with basophilic-directed phosphorylation sites, due to considerable competition with co-eluting peptides with multiple basic residues, resulting in a substantial number of unidentified basophilic peptides in IDA-based product ion spectral acquisition. Therefore, it is essential to identify yet-to-be-sampled phosphopeptides having multiple basic residues, thereby leading to mapped phosphorylation sites flanked experimentally to cope with basophilic-directed phosphorylation sites and the basophilic kinase–substrate relationships.

Liquid chromatography/mass spectrometry (LC/MS)–based phosphoproteomics remains challenging because of the low natural abundances (1–2% of the entire protein amount) of such species in complex biological samples [33]. The ability of multidimensional liquid chromatography (MDLC) to minimize sample complexity for bottom-up proteomics has played a pinnacle role in the advancement in peptide sequencing, protein identification, and quantification [34,35]. A high-/low-pH reversed-phase/strong anion exchange (RP–SAX–RP) system has been developed [36,37] and applied successfully to a phosphoproteomics analysis. This platform featured a high separation peak capacity and efficient retention of singly and multiply phosphorylated peptides. Considering, theoretically, that greater than 29% of human tryptic peptides have a neutral or basic net charge at a pH of less than 8.5, these peptides should not be retained efficiently by the SAX column. The viability of employing the three-dimensional (3D) RP–strong cation exchange (SCX)–RP platform in studies of anionic peptides is limited, however, by poor anion binding in the SCX column. Taking into consideration the advantages and drawbacks of RP, SCX, and SAX column chemistries, a platform incorporating both types of ion exchange columns should address the shortcomings of both RP–SAX–RP and RP–SCX–RP LC systems. Recently, we proposed an MDLC platform based on RP column chemistry complimented with SAX and SCX columns [38]. The inclusion of the complementary S(A/C)X column chemistries in the RP–SA(C)X–RP system allowed the retention of deprotonated peptides in the SAX trap column, followed by diversion of non-retained peptides to an online SCX trap column, thereby allowing identification of both anionic and cationic peptides from a single injection event. The RP–SA(C)X–RP platform provided more extensive protein and proteome coverage, thereby leading to improved protein quantification and protein tyrosine nitration identification from various complex biological samples. We suspected that a potential application of this RP–SA(C)X–RP platform would be the large-scale identification of anionic phosphorylation sites in which neutral hydroxyl groups have been replaced with negatively charged phosphoryl groups. In this study, we report the first global analysis of the *in vivo* phosphoproteome of the *M. fascicularis* cerebral cortex as a resource to the scientific community. The tissue lysate was subjected to in-solution trypsin digestion, followed by offline TiO_2 phosphopeptide enrichment and characterization of the purified peptides using a shotgun proteomics approach, involving an MDLC platform with RP and tandem anion/cation exchange columns [RP–SA(C)X–RP] coupled to mass spectrometric analysis [38].

To improve the confidence of phosphopeptide identification, a stringent secondary analysis using PhosphoRS 3.0 was applied to the mass spectrometric data [39]. This strategy yielded the characterization of 14,338 phosphopeptides, corresponding to 7572 distinct phosphosites from 2705 phosphoproteins. To the best of our knowledge, these results represent the largest compendium of the crab-eating macaque cerebral cortex phosphoproteome and will facilitate future biological investigations into the role of reversible phosphorylation in neuronal physiology and disease.

2. Experimental section

2.1. Materials and reagents

All solutions were prepared using ddH_2O generated from a Milli-Q water purification system (Millipore, Bedford, MA). Polyimide-coated fused-silica capillary (FSC) tubing was purchased from Polymicro Technologies (Phoenix, AZ). Jupiter C18 pack-