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Monitoring the native phosphorylation state of plasma membrane proteins from a single mouse cerebellum

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HIGHLIGHTS

- Phosphoproteomic strategies use various techniques to enrich for phosphopeptides.
- Sequential enrichment with various techniques enhances the number of identified phosphopeptides.
- We identified 1501 native phosphorylation sites of plasma membrane proteins.
- A bioinformatic screen identifies kinases which may be involved in the phosphorylation of identified phosphorylation sites.

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ABSTRACT

Neuronal processing in the cerebellum involves the phosphorylation and dephosphorylation of various plasma membrane proteins such as AMPA or NMDA receptors. Despite the importance of changes in phosphorylation pattern, no global phospho-proteome analysis has yet been performed. As plasma membrane proteins are major targets of the signalling cascades, we developed a protocol to monitor their phosphorylation state starting from a single mouse cerebellum. An aqueous polymer two-phase system was used to enrich for plasma membrane proteins. Subsequently, calcium phosphate precipitation, immobilized metal affinity chromatography, and TiO_2 were combined to a sequential extraction procedure prior to mass spectrometric analyses. This strategy resulted in the identification of 1501 different native phosphorylation sites in 507 different proteins. 765 (51%) of these phosphorylation sites were localized with a confidence level of 99% or higher. 41.4% of the identified proteins were allocated to the plasma membrane and about half of the phosphorylation sites have not been reported previously. A bioinformatic screen for 12 consensus sequences identified putative kinases for 642 phosphorylation sites. In summary, the protocol deployed here identified several hundred novel phosphorylation sites of cerebellar proteins. Furthermore, it provides a valuable tool to monitor the plasma membrane proteome from any small brain samples of interest under differing physiological or pathophysiological conditions.

1. Introduction

The cerebellum is one of the best investigated sub-structures in the brain. It is involved in fine tuning of motor control and plays a central role in motor learning. In addition, the cerebellum is important in non-motor functions such as language, cognition, pain, emotion, and addiction (Strick et al., 2009; Manto and Marmolino, 2009). In agreement with these observations, clinical reports and neuropsychological studies link cerebellar dysfunction to various forms of ataxia as well as attentional, cognitive, and affective deficits. Anatomically, the cerebellum is well structured. It receives input from various regions of the brain, mainly the brainstem and the spinal cord. The inputs are processed and integrated in the four cerebellar nuclei and in the cerebellar cortex. The well defined structure and function of the cerebellum have made it a model system to study synaptic transmission and temporal integration.

The proteins required for neural processing of the diverse motor and sensory information have been elucidated by various studies using microarrays (Evans et al., 2003; Lim et al., 2004; Xiao et al., 2005) or proteomic platforms (Schindler et al., 2006; Olsen et al., 2007; Becker et al., 2008; Selimi et al., 2009; Maurya et al., 2010). However, various proteins in the cerebellum undergo dynamic changes in their phosphorylation status (Qian et al., 2012; Widmer et al., 2003; Chung et al., 2003; Meissirel et al., 2011). This process is often intimately linked to the extensive synaptic plasticity of this brain area. Both long-term potentiation (LTP) and long-term

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depression (LTD) have been associated with learning and memory consolidation in the cerebellum. Indeed, LTD has been discovered in the cerebellum (Ito and Kano, 1982). This bidirectional modulation of plasticity is at the focus of many studies, as simple motor-learning paradigms are available to establish links between molecules and behaviour (De Zeeuw et al., 1998; Kishimoto and Kano, 2006; Evans, 2007). Synaptic plasticity in the cerebellum has therefore become an important model to study the molecular and cellular mechanisms of learning and memory (Evans, 2007). Electrophysiological and pharmacological studies have identified a variety of proteins participating in LTP and LTD, and many of the plastic processes require signalling by kinases and phosphatases. This has led to the identification of essential phosphorylation sites for LTP and LTD (Feil et al., 2003; Leitges et al., 2004; Boyden et al., 2006; Hansel et al., 2006). However, the majority of native phosphorylation sites in the cerebellum are still unknown.

Systems biology of physiological and pathophysiological conditions in the cerebellum, however, requires global analyses of phosphorylation sites. The native phosphorylation state of proteins can be monitored by various proteomic strategies (reviewed in: (Reinders and Sickmann, 2005; Thingholm et al., 2009). A serious drawback in these studies is the requirement of huge amounts of proteins. In addition, plasma membrane proteins which are at the focus of many physiological studies represent quantitatively only a minor fraction of the cellular proteins (Evans, 1991). Thus, we aimed at developing a proteomic strategy which enables the analysis of phosphorylation sites of plasma membrane proteins from a single cerebellum. This will minimize the number of animals required for each study. The successful implementation of the protocol was demonstrated by the identification of many known and unknown phosphorylation sites.

2. Materials and methods

2.1. Enrichment of plasma membranes

A protocol based on aqueous polymer two-phase systems (Schindler et al., 2008) was adapted to cope with the inclusion of protease and phosphatase inhibitors. All procedures were performed at 4 °C. 150 mg of mouse cerebellum were homogenized in 1.35 g of a two-phase system, composed of 6.89% (w/w) Dextran T500; 6.89% (w/w) polyethylene glycol 3350; 55.56 mM Tris/SO_4^{2-}, pH 7.8; 0.22% (w/w) iodoacetamide, 1 tablet per 5 ml of complete mini protease inhibitor (Roche, Mannheim, Germany) and PhosStop phosphatase inhibitor (Roche). A total of 50 strokes were applied in a glass-Teflon homogenizer with rotation at 500 rpm followed by 15 aspirations through a 20 gauge needle. The homogenate was centrifuged at 750 ×× *g* to separate the phases. The resulting top phase was transferred to a fresh bottom phase of 1.5 g of a two phase system, containing the following components: 6.2% (w/w) Dextran T500; 6.2% (w/w) polyethylene glycol 3350; 50 mM Tris/SO_4^{2-}, pH 7.8; 0.05% (w/w) iodoacetamide; 1 tablet per 5 ml of complete mini protease inhibitor and PhosStop phosphatase inhibitor. The top phase of this two-phase system was used to re-extract the bottom phase of the first two-phase system. To this end, both new two-phase systems were vortexed and subsequently centrifuged at 750 × *g*. After phase separation, top phases were transferred one bottom phase along; the bottom phase of the initial two-phase system was re-extracted with fresh top phase. All two-phase systems were vortexed and centrifuged at 750 × *g*. The extractions were repeated until the initial top phase was re-extracted 9 times. Plasma membranes were recovered from the last 3 top phases by diluting them 5-fold with 50 mM Tris/SO_4^{2-}, pH 7.8 followed by ultracentrifugation for 1 h at 100,000 × *g*. Subsequently, the pellet was washed 3 times with 2.5 M KCl and 3 times with 0.2 M Na_2CO_3 to remove peripheral plasma membrane proteins.

Enrichment of plasma membranes by the two-phase partitioning protocol was estimated by the activity of 5′-nucleotidase, a common marker for plasma membranes (Ramirez et al., 2004). The activity of 5′-nucleotidase was determined as described by Heppel and Hilmore (1951). Protein amounts were determined by the method of Bradford (1976).

2.2. Enrichment of phosphopeptides

Proteins were solubilized in 7 M urea, 2 M thiourea, reduced with 15 mM dithiothreitol for 30 min at room temperature followed by alkylation with 15 mM iodoacetamide for 30 min at room temperature in the dark. The protein solution was digested with Lys-C for 4 h at room temperature and subsequently diluted 6-fold with water. Trypsin was added and the sample digested at 37 °C over night. Phosphopeptides were extracted from this solution by the combination of three different techniques for phosphopeptide enrichment: calcium phosphate precipitation (CPP) (Zhang et al., 2007), IMAC (Ye et al., 2010), and TiO_2 (Thingholm et al., 2008).

2.3. Calcium phosphate precipitation

For calcium phosphate precipitation (CPP), the peptide solution was brought to 250 μl with water. 10 μl of 0.5 M Na_2HPO_4 and 10 μl of 2 M $NH_3{\cdot}H_2O$ were added and pH was adjusted to 10. 10 μl of 2 M $CaCl_2$ was added to precipitate phosphopeptides. The precipitate was pelleted by centrifugation for 10 min at 20,000 × *g*. The resulting supernatant was stored for further enrichment of non-precipitated phosphopeptides via TiO_2. The pellet was washed with 30 μl of 80 mM $CaCl_2$ and finally dissolved in 10 μl of 5% formic acid.

2.4. Immobilized metal ion affinity chromatography (IMAC)

Prior to IMAC enrichment, the solubilized peptides from the calcium phosphate precipitation procedure were loaded on a Poros R3 microcolumn and washed with 20 μl of 5% formic acid to desalt the sample (Gobom et al., 1999). Peptides were eluted from the column with 60% acetonitrile, 0.6% acetic acid and were incubated with 10 μl of Fe^{3+}-NTA for 1 h at room temperature. The IMAC material was washed twice with 50 μl of 60% acetonitrile, 0.6% acetic acid. Phosphopeptides were eluted with 30 μl of 2 M $NH_3{\cdot}H_2O$.

2.5. Titanium dioxide chromatography

Titanium dioxide (TiO_2) microcolumns were prepared by plugging a gel loader tip with an 8M Empore C8 extraction disc. TiO_2 beads were resuspended in 100% acetonitrile and were loaded onto the microcolumn. Phosphopeptides were diluted 5 times with 1 M glycolic acid, 80% acetonitrile, 5% trifluoro acetic acid and loaded onto the microcolumn. The sample was washed with 10 μl of 1 M glycolic acid, 80% acetonitrile, 5% trifluoro acetic acid followed by 30 μl of 80% acetonitrile, 5% trifluoro acetic acid. The phosphopeptides were eluted with 50 μl of 2 M $NH_3{\cdot}H_2O$ followed by 1 μl of 30% acetonitrile.

2.6. Nano-LC–MS

LC–MS/MS analyses were performed on a nanoliter flow EasyLC system (Thermo Fisher Scientific) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptides from the different enrichment steps were vacuum-dried, dissolved in 1 μl of 100% FA and 4.5 μl of water were added. 5 μl of the dissolved peptides was loaded onto a fused silica column (18 cm length, 100 μm inner diameter, 375 μm outer diameter, ReproSil, C18 AQ