主論文の要旨

KANPHOS: A Database of Kinase-Associated Neural Protein Phosphorylation in the Brain

（KANPHOS：脳神経系タンパク質リン酸化シグナルデータベース）

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【Introduction】

Protein phosphorylation and dephosphorylation are critical post-translational modifications in the regulation of cell signaling networks, especially in the nervous system, where these mechanisms contribute to brain development, functions and disorders. The levels of protein phosphorylation are regulated by the combined actions of protein kinases and phosphatases. With the advancement of mass spectrometry (MS)-based proteomics approaches, a variety of databases have been developed to document experimentally verified in vivo and/or in vitro phosphorylation sites from numerous biological samples. Several phos-phoproteomics databases have been developed, including UniProtKB, PhosphoELM, PhosphoSitePlus, PHOSIDA, SysPTM, jPOST, and Human Protein Reference Database (HPRD). However, none of these phosphoproteomics databases focus on brain phosphorylation events, and they fail to explain the upstream signaling events for phosphorylation because the identities of the kinases that phosphorylate the listed sites remain largely unknown. In this study, we developed and presented an online database named KANPHOS (Kinase-Associated Neural PHOspho-Signaling), which provides phosphorylation signaling information, including data on phosphoproteins, phosphorylation sites and responsible kinases, in the brain. Moreover, we performed kinase-oriented substrate screening (KIOSS) to obtain novel phosphoproteomics data for adenosine-A2a-receptor signaling, including downstream MAPK-mediated signaling, in the striatum/nucleus accumbens and registered them in KANPHOS.

【Materials and Methods】

System Configuration and Application Software:

The KANPHOS database is designed as a web-based database that is accessible from a web browser to allow simple and widespread use by researchers. It was built based on the neuroinformatics platform system named XooNIps (XOOPS module for Neuroinformatics) an extended module of the extensible object-oriented portal system (XOOPS) (https://xoops.org) which is an OS-independent content management system (CMS) web application written in PHP (Hypertext Preprocessor, https://www.php.net) In KANPHOS, XooNIps runs on CentOS (https://www.centos.org) using Apache (The Apache HTTP Server Project,: https://httpd.apache.org) as the web server and MySQL (https://www.mysql.com) as the da-tabase management system.

Coronal Slice Preparation and Phosphoproteomic Analysis:

Coronal brain slices (350 μm) were prepared from male C57BL/6 mice using a VT1200S vibratome. After dissecting of the striatum/NAc, slices were incubated at 30 °C in Krebs-HCO_3^- buffer. Coronal slices were pretreated with the indicated inhibitors (D2R agonist quinpirole, MAPK 1/3 inhibitor U0126) and then stimulated with the indicated activators

(A2AR agonist CGS21680, phosphatase inhibitor okadaic acid). After drug treatment, slices were immediately frozen in liquid nitrogen and cell lysate were collected. Phosphoprotein were enriched by GST-14-3-3ζ pulldown, followed by elution using guanidine solution, reduction, alkylation, demineralization, concentration and finally digestion with trypsin. Phosphopeptide was enriched by TiO_2 column and were analyzed by liquid chromatography/mass spectrometry (LC/MS) using an Orbitrap Fusion mass spectrometer.

【Results】

To construct the KANPHOS database of protein phosphorylation modification in the brain, we integrated data mainly from our own developed phosphoproteomic method (45%), from the literature (37%) and from Protoarray (18%) (Figure 1A, 1B, 1C). To obtain kinase-oriented protein phosphorylation information, we developed the Kinase-Interacting Substrate Screening (KISS) method for in vitro phosphoproteins and Kinase-Oriented Substrate Screening (KIOSS) method for in vivo phosphorylation dependent signaling. The ratio of protein phosphorylation sites identified using the KISS method was 36%, whereas the KIOSS method identified 9% of the total entries (Figure 1D). Although data from different species were included in KANPHOS, most of the phosphorylation events listed here are from rodents. The three species with the highest levels of phosphoproteins (phosphorylation sites) were humans (2010 (3782)), rats (1452 (4520)), and mice (680 (1662)) (Figure 1E).

To examine how the KANPHOS database works, we performed KIOSS to obtain phosphoproteomic data downstream of the adenosine signaling pathway in the striatum/NAc. We used this study as a model to demonstrate the ability of the KANPHOS database to identify novel phosphorylation-dependent signaling dynamics in the brain. To identify novel phosphoproteins downstream of adenosine-A2AR signaling, mouse striatum/NAc slices were treated with the A2AR agonist CGS21680 and the D2R agonist quinpirole (Figure 2A, 2B). PKA activity was confirmed by monitoring the phosphorylation level of well-known PKA substrates (Rap1gap at S563, and Rasgrp2 at S116/117) by immunoblotting. Next, we performed phosphoproteomic analysis using the KIOSS method, and identify almost 80% of the phosphoproteins enhanced by A2AR agonists were inhibited by D2R agonists; thus, these phosphoproteins represent candidate substrates of adenosine signaling. We next performed the KIOSS approach to explore MAPK-mediated signaling. The striatum/NAc slices were treated with a phosphatase inhibitor (okadaic acid) and/or a MEK inhibitor (U0126). Treatment of the slices with okadaic acid increased the phosphorylation of MAPK1/3 at T202/Y204, whereas pretreatment with a MEK inhibitor (U0126) reduced this okadaic acid mediated phosphorylation (Figure 2C). The okadaic acid treatment induced the phosphorylation of huge numbers of proteins, including MAPK

substrates, and pretreatment with U0126 specifically inhibited MAPK-mediated phosphorylation. Thus, the comparison between the treatments with okadaic acid and okadaic acid with U0126 identified more than hundreds of MAPK candidate substrates. Comparison of MAPK candidate substrates with A2AR downstream phosphoproteins identified 22 phosphoproteins that were phosphorylated by MAPK downstream of adenosine signaling (Figure 2D, 2E).

Here, we propose a workflow for analyzing MAPK phosphoproteomics data using KANPHOS pathway analysis as a model study. As we wanted to focus on A2AR signaling, we began with the neurotransmitter section by choosing dopamine “Pathways” icon on the home page, which opened the pathway viewer image of dopamine signaling in dopamine neurons (Figure 7A). MAPK was selected from the D2R-MSN (A2A-R) signaling pathway, and 17 phosphoproteins were found to be candidate substrates of MAPK downstream of A2AR signaling (Figure 7B). To obtain more information on the identified candidate substrates, the user can perform pathway analysis by clicking on the upper right corner of the substrate list to reveal the associated Gene Ontology terms and found several channels proteins in this process (Figure 7C). To further find which phosphoproteins are involved in this channel regulation process downstream of A2AR, next we go to the advance search option and from which we select Kinase (MAPK), signal drug (A2AR Agonist) and GO term (Channel) (Figure 7D). Here we revealed that hyperpolarization-activated cyclic-nucleotide gated (Hcn2, Hcn3), and voltage-dependent R-type Calcium channel Alpha 1E subunit (Cacna1e) proteins are involved in the MEK-MAPK pathway downstream of A2AR (Figure 7E).

【Conclusion】

To understand the biological role of phosphorylation, it is necessary to characterize up-stream signal transduction, including the kinases responsible for phosphorylation and the functional changes in downstream molecules. Phosphorylation signaling cascades vary among tissues, and completely different cellular reactions can result from the same protein phosphorylation event in different tissues due to slight differences in the cascade components. Our KANPHOS database is designed to refer not only to phosphorylation and the responsible kinases but also to related diseases, genetic polymorphisms, and knockout mice. By examining these associated data and pathway analysis of the protein phosphorylation selected in the KANPHOS search results, the signaling cascades participating in the phosphorylation of the target protein can be predicted. Compared to the other conventional databases, brain-specific, signal transduction and kinase-oriented phosphorylation data collection is unique to KANPHOS, and would provide high quality data with good usability for neuroscientists.