主論文の要旨

Phosphoproteomic analysis using the WW and FHA domains as biological filters

（WW ドメインおよび FHA ドメインを生物学的フィルターとして用いたリン酸化プロテオミクス解析）

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【Background】

As one of the most common post-translational modifications in eukaryotic cells, protein phosphorylation regulates almost every biological event. Liquid chromatography tandem mass spectrometry (LC-MS/MS)-based phosphoproteomics has become an indispensable tool for identifying global phosphopeptides from complex protein samples. However, the transient nature of protein phosphorylation state and the low relative abundance of phosphoproteins compared to the highly abundant structural or housekeeping proteins, make them difficult to identify using LC-MS/MS. Previously, we used 14-3-3ζ to selectively enrich for HeLa cell lysate phosphoproteins. However, because 14-3-3 does not isolate phosphoproteins lacking the 14-3-3-binding motif, I looked for other domains that could complementarily enrich for phosphoproteins. I here assessed and characterized the phosphoprotein binding domains Pin1-WW, CHEK2-FHA, and DLG1-GK. This study showed that unlike 14-3-3, which primarily isolates cytoplasmic and membrane phosphoproteins, WW and FHA can efficiently enrich nuclear and membrane-enclosed lumen phosphoproteins from HeLa cells treated with phosphatase inhibitors. These domains could enrich specific kinase oriented candidate substrates such as protein kinase A (PKA).

【Materials and Methods】

For pull-down assay, HeLa cells were treated with the phosphatase inhibitor calyculin A (50 nM until cells started to round up, approximately 9 min), okadaic acid (1 μM for 30 min), or a mixture of the PKA activator forskolin (20 μM) and the phosphodiesterase inhibitor IBMX (125 μM) for 30 min. Lysis buffer was applied immediately after washing and cellular proteins were extracted. Phosphorylated proteins were pulled down with 500 pmol of GST-WW, -FHA, -GK or -14-3-3ζ. For mass-spectrometry sample preparation, the bound proteins were eluted using guanidine solution (7 M guanidine and 50 mM Tris/HCl), and the eluate was subjected to reduction, alkylation, demineralization, concentration and finally digestion with trypsin. All LC-MS/MS experiments were performed using Q-Exactive mass spectrometry. Analysis of the raw data was performed with MaxQuant software.

【Results】

To enrich phosphoproteins, HeLa cells were treated with calyculin A (PP1 and PP2A inhibitor) or okadaic acid (PP2A inhibitor mainly) and subjected to GST affinity chromatography with Pin1-WW, CHEK2-FHA, DLG1-GK, and 14-3-3ζ. Silver staining (Fig. 1A) and immunoblotting data (Fig. 1B) showed that calyculin A and okadaic acid treatment induced an increase in the number and amount of phosphoproteins. CHEK2-FHA and Pin1-WW precipitated different phosphoproteins than 14-3-3ζ, suggesting these two domains are good tools for identifying diverse signaling substrates such as PKA, MAPK/CDK, ATM/ATR, and AKT. Compared to Pin1-WW, CHEK2-FHA, and 14-3-3ζ, DLG1-GK did not

demonstrate strong phosphoprotein binding in the drug-treated samples. The proteins bound to WW, FHA, GK, or 14-3-3ζ were identified by LC-MS/MS analysis. Using WW, FHA, GK, and 14-3-3ζ, I identified 46, 156, 14, and 146 phosphoproteins, respectively, whose phosphopeptide intensities in calyculin A-treated cells were more than five times those in control cells in at least two independent experiments (Fig. 2A). The Venn diagram shows that 26, 114, and 107 phosphoproteins were specifically associated with WW, FHA, and 14-3-3ζ, respectively (Fig. 2B). Subcellular localization based on GO classification showed that, in comparison with 14-3-3ζ, WW and FHA preferentially bound to nuclear and membrane-enclosed lumen phosphoproteins (Fig. 2C). Sequence alignment of the phosphopeptides showed that WW preferentially bound to proline-rich (pS/pT)P motifs. FHA bound to the (pS/pT)P motif with preference for E/I/D in the +3 position and basic amino acids in the -3 position, whereas 14-3-3ζ prefers RXXpS (where X is any amino acid) (Fig. 2D). KEGG pathway analysis from the phosphoproteins bound to these domains showed that both WW and FHA could bind to phosphoproteins involved in the cell cycle and cancer development (Fig. 2E). FHA also tends to bind phosphoproteins involved in RNA transport, oocyte meiosis, ubiquitin-mediated proteolysis, and Wnt signaling pathway, indicating a possible role for this domain in multi-signaling complexes (Fig. 2E). Based on the data from the phosphatase inhibitor calyculin A, I employed WW and FHA, in addition to 14-3-3ζ, to enrich PKA signaling substrates and I identified 26, 46, and 36 phosphoproteins responding to FSK/IBMX associated with these baits, respectively (Fig. 3B). Sequence alignment of the FSK/IBMX-stimulated phosphopeptides bound to WW, FHA, and 14-3-3ζ showed similarity with the PKA phospho-motif sequence suggesting these domains bind PKA signaling substrates (Fig. 3D). To examine whether the candidate substrates are phosphorylated by PKA *in vivo*, I focused on TNKS1BP1, the 182 kDa Tankyrase 1 binding protein 1. Immunoprecipitation analysis using anti-TNKS1BP1 antibody and immunoblotting with PKA phospho-substrates motif antibody showed that TNKS1BP1 phosphorylation was increased in FSK/IBMX-treated cells and reduced upon H-89 exposure (Fig. 3E).

【Discussion】

In this study, I characterized the phosphoprotein binding potential of several domains such as WW, FHA, GK, and 14-3-3. Silver staining data (Fig. 1A) showed clear enhancement in protein levels in phosphatase inhibitor-treated samples in the case of WW, GK, and 14-3-3ζ, suggesting these proteins undergo phosphorylation state-dependent binding to the bait domains. Because the FHA domain bound to so many proteins, I could not distinguish the enhancement of phosphorylation-dependent protein binding. However, immunoblotting with various kinase phospho-substrate motif antibodies demonstrated that WW and FHA, together with 14-3-3, bound to many phospho-proteins (Fig. 1B). From the phosphoproteomic data, I found 46, 156, 14, and 146 phosphoproteins bound to WW, FHA, GK, and 14-3-3,

respectively, upon calyculin A stimulation. Analysis of subcellular localization (Fig. 2C) showed that the WW and FHA domains primarily bound nuclear and luminal membrane phosphoproteins, whereas 14-3-3 bound cytoplasmic and membrane phosphoproteins. These data potentially reflect the subcellular localization of the physiological binding partners since both Pin1 (with a WW domain) and CHEK2 (with a FHA domain) work mainly in nucleus *in vivo*. Consistent with a previous report that the WW and FHA domains are crucial regulators of cell cycle progression and the DNA damage response, KEGG pathway analysis (Fig. 2E) showed that both WW and FHA are capable of precipitating phosphoproteins involved in the cell cycle and cancer development. These data suggest these domains may be used to identify biologically functional phosphoproteins. To capture PKA signaling molecules, I found twelve of the substrates (CAMKK1; CTNNB1; DSP; MARK3; NCOR1; NEDD4L; PDE3A; PDE3B; PFKFB2; RAF1; TPR; TRIM32) obtained with these baits had already been reported as PKA substrates. Alignment of candidate phosphopeptide sequences clearly indicated that the WW and FHA domains as well as the 14-3-3 protein retain the PKA motif after FSK/IBMX stimulation (Fig. 3D). These data illustrate the utility of these domains in identifying substrates of particular kinases such as PKA. From these proteomic data, I identified TNKS1BP1 as a PKA candidate substrate that was obtained from WW-bound phosphoproteins, following FSK/IBMX stimulation. I examined the phosphorylation of TNKS1BP1 by PKA upon FSK/IBMX stimulation in HeLa cells, and the immunoprecipitation data (Fig. 3E) suggest that TNKS1BP1 is a PKA substrate.

【Conclusions】

In summary, this approach of identifying kinase oriented substrates screening (KiOSS) using the phosphoprotein-binding domains WW and FHA provided many novel substrates, including known substrates that are mainly nuclear or enclosed in the luminal membrane. Together, these data suggest that the WW and FHA domains serve as biological filters that may be used to identify phosphosignaling downstream of a specific kinase.