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Large-scale identification of novel mitosis-specific phosphoproteins

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ABSTRACT

Systematic identification of phosphoproteins is essential for understanding cellular signalling pathways since phosphorylation plays important roles in cellular regulation. Monoclonal antibody MPM-2 recognizes a discrete set of mitosis-specific phosphoproteins and constitutes a specific tool to investigate the significance of phosphorylation in cell cycle. However, due to the difficulties in identifying antigens revealed on immunoblot membrane, only minority of MPM-2 antigens have been identified. Here we originated proteomics approaches for large-scale identification of MPM-2 phosphoproteins. Mitotic extracts were run on several two-dimensional gel electrophoresis (2D) in parallel, and stained by Coomassie Blue. Each individual spot on one of the gels was excised, and proteins in it were further resolved by regular SDS-electrophoresis and blotted on membrane for MPM-2 stain. Counterparts of the positive proteins were selected on another parallel 2D gel and identified by mass-spectrometry. Using this strategy, 100 spots were excised from Coomassie-stained 2D gel and screened by 1D immunoblots for MPM-2 reactivity, and 22 proteins containing potential MPM-2 epitope were identified in addition to a known MPM-2 antigen, laminin-binding protein. These results were further validated by immunofluorescence, co-immunoprecipitation and in vitro phosphorylation assay. The identification of an unprecedented number of potential MPM-2 phosphoprotein antigens gives new insight into the range of proteins involved in the regulation of the early stages of cell division. Meanwhile, this strategy could be used wherever unknown antigens are explored, especially for antibodies that can recognize more than one antigen.

1. Introduction

Protein phosphorylation has been generally recognized as a fundamental mechanism in cellular procession regulating, including the regulation of cell-cycle transition from interphase to mitosis. There is a dramatic accumulation of phosphoproteins at the entry of mitosis, accomplished by dynamic equilibrium between the activities of protein phosphatases and protein kinases [1,2]. Large-scale identification of either a complete or arbitrarily chosen set of phosphoproteins, which are postulated to have roles in mitosis, will certainly enhance our understanding of phosphorylation-dependent mitotic regulation network.

Over 50 proteins become specifically phosphorylated during the G2/M transition and react with monoclonal antibody MPM-2, which was generated by using M-phase Hela cells as an immunogen [3]. It is believed that phosphorylation at MPM-2 antigenic sites regulates mitotic events since microinjection of MPM-2 into oocytes inhibited mitosis entry and exit [4]. This functional significance of MPM-2 antigens is reinforced by the fact that MPM-2 antigens have been found to decorate some important mitotic apparatuses, such as centrosome, kinetochore, mitotic spindle, chromosome axis, midbody and Golgi complex [5–7], suggesting that the structural rearrangements observed during mitosis are controlled by phosphorylation events of MPM-2 epitopes. Most of the known MPM-2 antigens have crucial and versatile M-phase functions. They include microtubule associated-proteins MAP-1 and MAP-4 [8,9], DNA topoisomerase IIα and β[10], p42mapk [11], Cdc25 phosphatase [12], Cdk1-inhibitory Wee1 and Myt1 kinases [13–15], NIMA kinase [16], Cdc27 component of the anaphase-promoting complex (APC) [17], p54nrb [18] and SREBP1 [19]. It is now very clear that MPM-2 antigens are important mitotic regulators and effectors. Therefore, MPM-2 constitutes a specific tool to screen the important mitotic factors, which are phosphorylated exclusively during the G2/M transition. Definition of more MPM-2 antigens would contribute to our overall understanding of the phosphorylation-dependent cellular regulation mechanism.

Many researches have tried to identify mitotic MPM-2 phosphoproteins in large scale. Joanne M. Westendorf has originated expression cloning technique for isolating cDNAs encoding MPM-2 reactive proteins that are efficient substrates for M-phase kinases [7,20]. Proteins from phage plaques were phosphorylated by M-phase kinases and screened for MPM-2 binding. Finally, cDNAs for 10 MPM-2 reactive proteins were cloned. P. Todd Stukenberg developed two complementary approaches to identify mitotic phosphoproteins. Proteins were translated from pools of cDNA plasmids in vitro and were screened by either MPM-2 recognition or electrophoretic mobility shifts. In this assay, the authors identified 20 different proteins that are phosphorylated in a mitosis-specific manner, and at least six of them were true

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MPM-2 antigens [21]. After 20 years of continuous exploration, only minority of the MPM-2 antigens have been identified. The major obstacle was the impracticability of identifying the antigens revealed on immunoblot membrane. The same problems exist in the antigen identification for other antibodies, such as determinations of autoantigens for serum antibodies from some diseases. Many efforts have been made to resolve this difficulty. Shimadzu Corporation had constructed a system by which microscale on membrane PMF analysis can be conducted on proteins transferred to a PVDF membrane [22]. However, its eventual application was limited because high cost was required for materials and facilities.

Obviously, systematic identification of the large set of mitotic MPM-2 phosphoproteins revealed on immunoblot membrane remained an interesting but unsolved problem. However, recent developments of proteomics techniques inspired us to explore its application in this field. We have originated proteomics methods to identify MPM-2 reactive proteins in large scale, but only three novel MPM-2 antigens were confirmed in this manner [23]. The primary challenge was to match spots or bands revealed on the immunoblot membrane and Coomassie Blue (CBB)-stained gel accurately, since their whole patterns and intensity were very different (see Discussion). In the present paper, we improved our original proteomics strategy and presented a new and much more precise way for systematic and broadly applicable screen for MPM-2 reactive proteins from extracts of mitotic Hela cells. We demonstrated this technique by identifying 22 proteins (100 protein spots were screened) containing potential MPM-2 epitope. Furthermore, for 3 of the 22 proteins, their reactivity with MPM-2 was confirmed by immunofluorescent co-localization, immunoprecipitation and in vitro phosphorylation assay. It is likely that our methods for large-scale identification of mitosis-specific phosphoproteins will generate new probes for understanding of the process of cell division.

2. Materials and methods

2.1. Cell cultures and synchronization

Hela cells and CHO cells were cultured in Dulbecco's modified Eagles medium (DMEM, Gibco) supplemented with 10% (v/v) fetal calf serum at 37 °C in 5% CO_2. To arrest cells in mitosis (in fact at G2/M transition), cells were treated with 40 ng/ml nocodazole (Sigma) for 16 h. To arrest cells in interphase (in fact at G1/S transition), cells were treated with 5 mM hydroxyurea for 16 h.

2.2. 1D immunoblots to screen protein spots from 2D gels

Firstly, mitotic extracts were prepared from Hela cells and resolved by several 2D gels. Briefly, mitotic Hela cells were washed three times with 250 mM Sobitol containing 10 mM Tris–HCl (pH7.0) and directly solubilized in extraction buffer (8 M urea, 4% CHAPS, 40 mM Tris, 1% dithiothreitol, 0.1 mM PMSF, 5 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin, 1 μM microcystin-LR (Calbiochem), 0.2 mM sodium orthovanadate), swollen on ice for 30 min and homogenized by ultrasonic. After 15,000 rpm centrifugation for 45 min at 4 °C to remove debris, the protein content of the supernatant was calculated using Bradford protein assay (BioRad) and then frozen the extracts at −80 °C. When we performed 2D-SDS-PAGE according to the manufacturer's recommendation, 1 mg solubilized samples were applied to Immobiline Drystrips (18 cm, pH 3~10, Amersham Pharmacia Biotech) overnight at room temperature (RT) to ensure maximal diffusion of the protein into the strip. The strips were then loaded onto an Ettan IPGphor II unit (Amersham Pharmacia Biotech) and covered with a layer of Drystrip cover fluid (Amersham Pharmacia Biotech). IEF was carried out as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V gradient for 30 min, and 8000 V for 60,000 Vh. After IEF, the DryStrip cover fluid was removed and the strips were equilibrated in 2% SDS, 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue supplement with 10 mg/ml dithiothreitol and 25 mg/ml iodoacetamide respectively for 15 min on a rocking table. After the equilibration, IEF strips were drained and placed on top of a continuous vertical SDS-PAGE gel (11.8% acrylamide, 0.2% bisacrylamide; BioRad). Standard SDS-PAGE was performed and gels were stained by CBB or transferred to nitrocellulose (NC) membrane (Millipore).

Secondly, every protein spot excised from 2D gels was screened by 1D immunoblots. Every distinguishable spot was excised from three 2D gels respectively as precisely as possible with a clean razor blade. The spots were extensively washed with several changes of Millipore water, destained in 25 mM NH_4HCO_3, 50% acetonitrile and finally dried in 100% acetonitrile. The dried gel-spots were incubated in SDS sample buffer (50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min. Three correlated spots were pooled and resolved again in one track of regular SDS-PAGE gels (11.8% acrylamide, 0.2% bisacrylamide; BioRad), and transferred to NC membrane. The blots were blocked with 3% bovine serum albumin (BSA) diluted in TBST (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, containing 0.05% Tween-20) for 60 min at RT and probed overnight with monoclonal antibody MPM-2 (gift from Dr. Jian Kuang) or rabbit anti-NPM antibody (made in our lab) at 4 °C. The membranes were washed 3 times with TBST, and an alkaline phosphatase-conjugated horse anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc) and NBT/BCIP (Promega) were used for development.

2.3. MALDI-QTOF MS analysis

CBB-stained MPM-2-reactive spots were excised from 2D gel, destained and subjected to in-gel trypsin digestion as manufacturer's instruction. Briefly, an aliquot (1 μl) of the peptide extract was mixed with 1 μl saturated solution of α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml) prepared in TFA (0.1% trifluoroacetic acid, 50% acetonitrile) and submitted to MALDI-QTOF mass-spectrometry using Qstar Pulser I

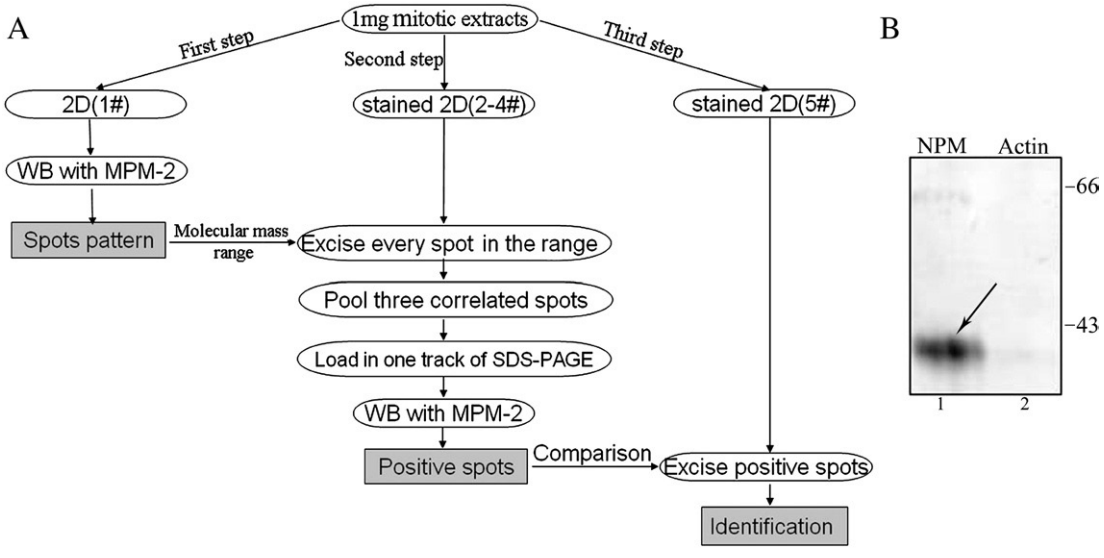

Fig. 1. A novel approach for identifying unknown antigens with existing antibodies. (A) Flow diagram of the procedure. 1 mg mitotic extracts were resolved by five 2D gels in parallel. 2#–5# 2D gels were stained. Firstly, proteins on 1# 2D gel were subjected directly to western blotting with MPM-2, revealing a MPM-2 reactive "spots pattern" on the NC membrane. Secondly, every spot within the molecular mass span of the "spots pattern" was cut out from 2# to 4# 2D gels. The three correlated spots were pooled, loaded into one track of 1D SDS-PAGE, and screened for MPM-2 binding. Finally, MPM-2 positive spots were selected on the 5# 2D gel and identified by MS. (B) Analysis of NPM and actin using this assay. Protein spots for NPM and actin were excised from 2D gel, resolved again by regular SDA-PAGE in track 1 and track 2 respectively and stained with anti-NPM polyclonal antibody. The arrow indicated the positive band. The position of molecular mass markers (in kDa) was shown on the right side.