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Parallel Assessment of Tyrosine Phosphorylation and Nuclear Targeting of Proteins

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ABSTRACT

Phosphotyrosine signaling plays a vital role in cell regulation—from receptor activation, through stimulation of signal networks and nuclear targeting, to final cellular responses. Here, we propose a new approach to monitor the spatial and temporal aspects of tyrosine phosphorylation and dephosphorylation. The method can be used to determine whether protein tyrosine phosphorylations and dephosphorylations occur

in the cytosol or the nucleus and to ascertain whether such modifications are associated with nuclear traffic. Promyelocytic leukemia (HL-60) cells are used as the experimental model. Biotinylated cytosolic proteins from donor cells are used to trace nuclear transport in permeabilized recipient cells. Thereafter, 2-D gel electrophoresis is applied to fractionate the cytosolic and nuclear proteins of the recipient cells, which are subsequently blotted onto polyvinylidene difluoride membranes. The membranes are developed with streptavidin and then reprobed with anti-phosphotyrosine antibodies. The major advantages of the protocol are that it is simple to perform, and reproducible results are obtained by overlaying the patterns of biotinylated and/or tyrosine-phosphorylated proteins. Moreover, several hundred cytosolic and nuclear proteins can be analyzed in parallel. Thus, by comparing the 2-D gel electrophoresis maps of biotinylated and tyro-

sine-phosphorylated proteins, it is possible to determine the involvement of trafficking of the latter proteins in cell signaling.

INTRODUCTION

Protein kinase cascades play an important role in the transduction of signals in response to extracellular stimuli (6) from the surface of a cell to the nucleus. Distinct spatial and temporal patterns of tyrosine phosphorylations occur in many proteins such as receptors, protein kinases, adaptor proteins, and transcription factors (6,7,9). Protein phosphorylation can influence gene expression in different ways: (i) activated protein kinases are translocated from the cytoplasm to the nucleus, where they phosphorylate target proteins (3,4,6); (ii) signaling protein adaptors and/or regulatory proteins are kept in a la-

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tent state in the cytoplasm and enter the nucleus only after being phosphorylated by cytoplasmic protein kinases (7,9); and (iii) tyrosine residues of nuclear proteins are dephosphorylated upon the cessation of extracellular stimuli (10,11).

Clearly, it would be of great value to know when and where proteins are tyrosine phosphorylated or dephosphorylated. However, to our knowledge, no method has addressed this question without using the radioactive markers ^{125}I - or ^{35}S -methionine and ^{32}P -orthophosphate (5). Therefore, we developed a novel, nonradioactive approach that can be used in any research laboratory to elucidate the spatial and temporal characteristics of protein tyrosine phosphorylation and nuclear targeting. Furthermore, we demonstrate the feasibility of applying the technique to analyze phosphotyrosine signaling and transport in proliferating human promyelocytic leukemia (HL-60) cells.

MATERIALS AND METHODS

Cell Culture

HL-60 cells were cultured in RPMI 1640 medium (Life Technologies, Täby, Sweden) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies) in 5% CO_2 at 37°C in a humidified incubator.

Isolation and Biotinylation of Cytosolic Proteins

The cytosolic fraction of proliferating HL-60 cells was prepared essentially as described by Adam et al. (1). In short, proteins in the fraction were biotinylated by exposure to 5 mM N-hydroxysuccinimide biotin (Sigma, St. Louis, MO, USA) for 30 min at 4°C. Thereafter, unbound biotin was removed by subjecting the cytosolic fraction to extensive dialysis (Spectra/Por® membrane tubing, molecular mass cut-off 3.5 kDa; Spectrum Medical Industries, Laguna Hills, CA, USA) against nuclear import buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM

EGTA, 2 mM DTT, 1 mM orthovanadate, 5 mM sodium fluoride, and 1 $\mu\text{g}/\text{mL}$ each aprotinin, leupeptin, and pepstatin). Aliquots of the cytosolic fraction were frozen and stored at -76°C.

Digitonin Permeabilization of HL-60 Cells and Assaying Nuclear Import

In short, the protocol proposed by Adam et al. (1) was modified as follows. The cells were harvested by low-speed centrifugation at $1500\times g$ and then washed twice with ice-cold PBS (pH 7.3) and once with import buffer. The cell pellet was then gently resuspended to 5×10^5 cells/mL in ice-cold

nuclear import buffer containing digitonin (Sigma) at a concentration of 30 $\mu\text{g}/\text{mL}$ (diluted from a 20 mg/mL stock solution in dimethyl sulfoxide). The cells were subsequently placed on ice for 5 min, and the reaction mixture was gently inverted once a minute. After incubation, the cells were centrifuged at $1500\times g$ for 7 min at 4°C and then washed at least three times with ice-cold import buffer to remove digitonin and prevent permeabilization of the nuclear membranes. Freshly digitonin-permeabilized HL-60 cells were used in the nuclear import experiments.

The permeabilized cells were mixed with cytosolic fraction and complete

Figure 1. Schematic diagram outlining the experimental procedures.

Figure 2. 2-D gel electrophoresis (2-DE) patterns of biotinylated and tyrosine-phosphorylated cytosolic (CYTOSOL) and nuclear (NUCLEUS) proteins isolated from proliferating recipient HL-60 cells after nuclear import. Digitonin-permeabilized recipient HL-60 cells devoid of their own cytoplasm were incubated with donor cytoplasm containing biotinylated cytosolic proteins. After allowing nuclear import, cytosolic and nuclear proteins were isolated, fractionated by 2-DE, and analyzed for streptavidin-HRP (biotinylation) or reactivity to anti-phosphotyrosine antibodies (tyrosine phosphorylation). Symbols: arrows, proteins tyrosine phosphorylated in the cytosol and then translocated into the nucleus; circles, proteins translocated into the nucleus and then tyrosine phosphorylated; triangles, proteins that were tyrosine phosphorylated in the cytoplasm and then dephosphorylated before or after translocation into the nucleus; squares, tyrosine-phosphorylated proteins residing permanently in the nucleus; diamonds, tyrosine-phosphorylated proteins residing permanently in the cytoplasm.

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Table 1. Location of Tyrosine Phosphorylation of Nuclear Proteins in Proliferating HL-60 Cells

Cell Compartment of Protein Tyrosine Phosphorylation	Symbol	Cytosol		Nucleus	
		Biotinylation	pY	Biotinylation	pY
1. Proteins tyrosine phosphorylated in the cytosol and subsequently translocated to the nucleus	↘	+	+	+	+
2. Proteins translocated into the nucleus and then tyrosine phosphorylated	o	+	-	+	+
3. Proteins tyrosine phosphorylated in the cytoplasm and then dephosphorylated before or after translocation into the nucleus	Δ	+	+	+	-
4. Tyrosine-phosphorylated proteins residing permanently in the nucleus	□	-	-	-	+
5. Tyrosine-phosphorylated proteins residing permanently in the cytoplasm	◇	+	+	-	-

Cytosolic proteins from donor cells were biotinylated and assessed after nuclear import into permeabilized recipient cells. The nuclear import was allowed to proceed for 30 min, and the cytosolic and nuclear proteins in the recipient cells were subsequently fractionated by 2-D gel electrophoresis. The proteins were then transferred to PVDF membranes and probed with streptavidin (Biotinylation) and anti-phosphotyrosine (pY) antibodies, respectively.

nuclear import buffer containing an energy-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 20 U/mL creatine phosphokinase). Nuclear import was allowed to proceed for 30 min at 30°C while being gently mixed by hand.

The integrity of the nuclear membrane and protein transport function was determined by optical sectioning with a confocal laser-scanning microscope (Sarastro® 2000; Molecular Dynamics, Sunnyvale, CA, USA). The microscope settings were optimized for the detection of the three different fluorescent probes we used to assess nuclear transport characteristics. These included the polyoma virus structural protein, VP1, which carries a nuclear targeting sequence (NLS), and BSA, which lacks NLS; both of these probes were labeled with fluorescein isothiocyanate (FITC). Cytosolic proteins that were biotinylated and subsequently marked with fluorescent streptavidin (streptavidin-ALEXA) constituted the third type of probe. When using the fluoresceinated proteins VP₁ and BSA, nuclei were

counterstained with propidium iodide. In experiments with the streptavidin-probed proteins, DNA was marked with SYTOX®-Green nucleic acid stain (Molecular Probes, Eugene, OR, USA). Isolated nuclei were microscopically pure, and imported proteins were found in the nuclei but not attached to it. We also confirmed that the import of cytosolic proteins occurred in the presence, but not in the absence, of ATP.

Isolation and Analysis of Cytosolic and Nuclear Proteins

Cytosols and nuclei were isolated according to Antalis et al. (2), with some modifications. Briefly, cells were collected, washed twice in PBS (pH 7.5), resuspended to 3×10^7 cells/mL in solution A (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 0.05 % Nonidet™ P-40, 1 mM PMSF, 1 mg/mL aprotinin, leupeptin, and pepstatin, 5 mM sodium fluoride, and 1 mM sodium orthovanadate), and then allowed to swell for 15 min at 0°C. Thereafter, the cell suspension was

shaken vigorously by hand and immediately mixed in a 1:1 ratio (v/v) with solution B (i.e., solution A supplemented with 0.6 M sucrose B). The cell homogenates were then centrifuged at 1500× g for 5 min. The supernatant, corresponding to the cytosolic fraction, was clarified by centrifuging at 15000× g for 15 min and then frozen at -76°C. Pelleted nuclei were washed twice by centrifuging at 1500× g for 5 min, first with a 1:1 mixture of solutions A and B and then three times with solution C [i.e., a 1:1 (v/v) mixture of solutions A and B without Nonidet P-40]. The nuclei were subsequently examined for purity and integrity under a light microscope and then frozen at -76°C.

Total nuclear proteins were prepared by mixing approximately 5×10^7 nuclei/mL in lysis solution [8 M urea, 4% CHAPS, 1% DTT, 0.8% 2-D pharmalytes (pH 3–10), and 1 mM PMSF] and then centrifuging at 15000× g for 30 min. 2-D gel electrophoresis, including isoelectric focusing and gel electrophoresis (SDS-PAGE), was performed according to the manufacturer's in-

structions (Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were subsequently blotted on Immobilon™ polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and probed with anti-phosphotyrosine antibody (mouse monoclonal IgG2b_k; Upstate Biotechnology, Lake Placid, NY, USA) or streptavidin-horseradish peroxidase (HRP) (Amersham Pharmacia Biotech). Enhanced chemiluminescence (ECL®) (Amersham Pharmacia Biotech) was used to detect immunoreactive bands.

RESULTS AND DISCUSSION

We used the experimental strategy described in Figure 1 to elucidate the location of proteins showing different degrees of tyrosine phosphorylation in proliferating HL-60 cells. Briefly, cytosolic proteins were isolated and biotinylated *in vitro*. Proliferating recipient HL-60 cells were then permeabilized with digitonin, and the cytosol was removed and replaced with the biotinylated cytoplasmic proteins of donor cells. Nuclear import was then allowed to proceed for a specific amount of time, and cytosolic and nuclear proteins in these recipient cells were isolated, fractionated by 2-D gel electrophoresis, and analyzed by ECL for reactivity to streptavidin and anti-phosphotyrosine antibody. More than 400 cytosolic and 300 nuclear peptides were detected by silver staining; both the electrophoretic patterns and the relative amounts of the proteins were highly reproducible (data not shown). Figure 2 illustrates the characteristic tyrosine phosphorylation of representative segments (30–100 kDa and 4–8.5 pI) of the total number of cytoplasmic and nuclear protein 2-D gel electrophoresis maps. To determine whether a certain tyrosine-phosphorylated cytosolic or nuclear protein is biotinylated, two ECL films obtained from the same PVDF membrane were probed with anti-phosphotyrosine antibodies and streptavidin, respectively, and then merged. In this way, five different cases of protein modifications could be detected: (i) biotinylation and tyrosine phosphorylation in both the cytoplasm

and the nucleus (arrows in Figure 2); (ii) biotinylation but not tyrosine phosphorylation in the cytoplasm, and both biotinylation and tyrosine phosphorylation in the nucleus (circles in Figure 2); (iii) biotinylation and tyrosine phosphorylation in the cytoplasm, but only a biotinylation signal in the nucleus (triangles in Figure 2); (iv) no biotinylation at all, and tyrosine phosphorylation only in the nuclei of recipient cells in which nuclear import had been allowed to proceed (squares in Figure 2); and (v) biotinylation and tyrosine phosphorylation only in the cytosol (diamonds in Figure 2). The conclusions we drew from these observations are summarized in Table 1, and the patterns of protein tyrosine phosphorylation revealed by 2-D gel electrophoresis are shown in Figure 2.

Concerning the spots denoted by triangles in Figure 2, there are other even more weakly biotinylated protein spots with detectable tyrosine phosphorylation in the nucleus fraction. Moreover, when the measurement time was extended 3- to 5-fold, still no signal was detected. We are therefore convinced that the absence of tyrosine phosphorylation is due to dephosphorylation and not to an effect of insufficient sensitivity of detection.

In the fourth case, the proteins apparently resided permanently in the nucleus because they were not found to be (but theoretically may well have been) biotinylated in the cytoplasmic fraction. An explanation for this might be that newly translated nuclear proteins are rapidly translocated from the cytoplasm, and, therefore, the relative amount of nascent nuclear proteins in the cytoplasmic fraction at the moment of extraction is very small. Moreover, it is possible that proteins from disassembled nuclei of mitotic cells contaminate the cytoplasmic fraction. However, such nuclear proteins should not interfere significantly with our method because less than 5% of the cells in a proliferating, asynchronous HL-60 population are mitotic (8).

In summary, our method represents a powerful new tool for studying spatial and temporal aspects of protein tyrosine phosphorylation and translocation of the proteins to the nucleus in different types of cells and physiological states.

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