



BRIEF COMMUNICATIONS

Identification of Immuno-Affinity Isolated Phosphotyrosine Proteins from WEHI-231 Cells

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Abstract: A goal of the Alliance for Cellular Signaling (AfCS) Protein Chemistry Laboratory is the identification of phosphoproteins in mouse B lymphocytes (B cells). In order to identify proteins phosphorylated on tyrosine, WEHI-231 cells were treated with pervanadate, a tyrosine phosphatase inhibitor, and tyrosine-phosphorylated proteins were enriched using anti-phosphotyrosine antibodies. The enriched proteins were resolved by SDS-PAGE. Proteins present in individual bands were excised and identified by liquid chromatography-tandem mass spectrometry. A total of 120 proteins were identified. Twenty-nine of these were previously characterized phosphotyrosine-containing proteins. The list included several SH2 domain-containing proteins and nine novel proteins.



Introduction

An important goal of the AfCS Protein Chemistry Laboratory is the global analysis of ligand-induced changes in protein phosphorylation. The first step in this process is the identification of known and novel phosphoproteins present in the AfCS model cell systems. Recent improvements in nanoscale liquid chromatography and mass spectrometry make it possible to identify proteins phosphorylated on serine and threonine residues on a proteome-wide basis (1). Identification of proteins phosphorylated on tyrosine has been more difficult due to their low abundance. However, antibodies against phosphotyrosine residues have been used to enrich phosphorylated proteins following activation of receptor tyrosine kinases (2,3). We used a similar approach to identify proteins phosphorylated on tyrosine following treatment of the WEHI-231 B-cell line with the tyrosine phosphatase inhibitor pervanadate. In this communication, we present the identification of proteins specifically isolated from pervanadate-treated cells with two separate anti-phosphotyrosine antibodies.

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Methods

Summary of procedure:

- Phosphoproteins were generated by treating WEHI-231 cells with pervanadate.
- Phosphotyrosine proteins were enriched by immunoprecipitation using phosphotyrosine antibodies PT-66 (Sigma-Aldrich) or P-Tyr-100 (Cell Signaling Technologies).
- Enriched proteins were separated on SDS gels and stained with Colloidal Blue.
- Individual bands were excised and proteins digested with trypsin.
- Proteins were identified by LC-MS/MS using a nanoscale C18 column coupled in-line with an ion trap mass spectrometer.
- The MS and MS/MS data were used to search NCBI mouse and mammalian nonredundant protein databases to identify proteins.

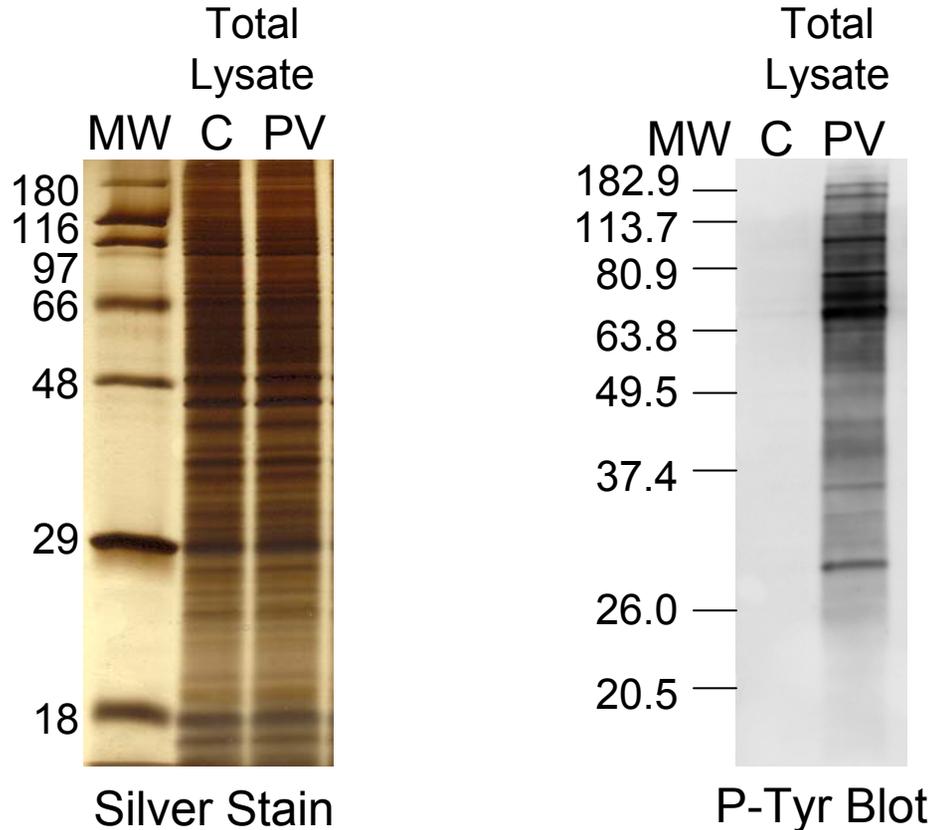
See [detailed methods](#).



Results

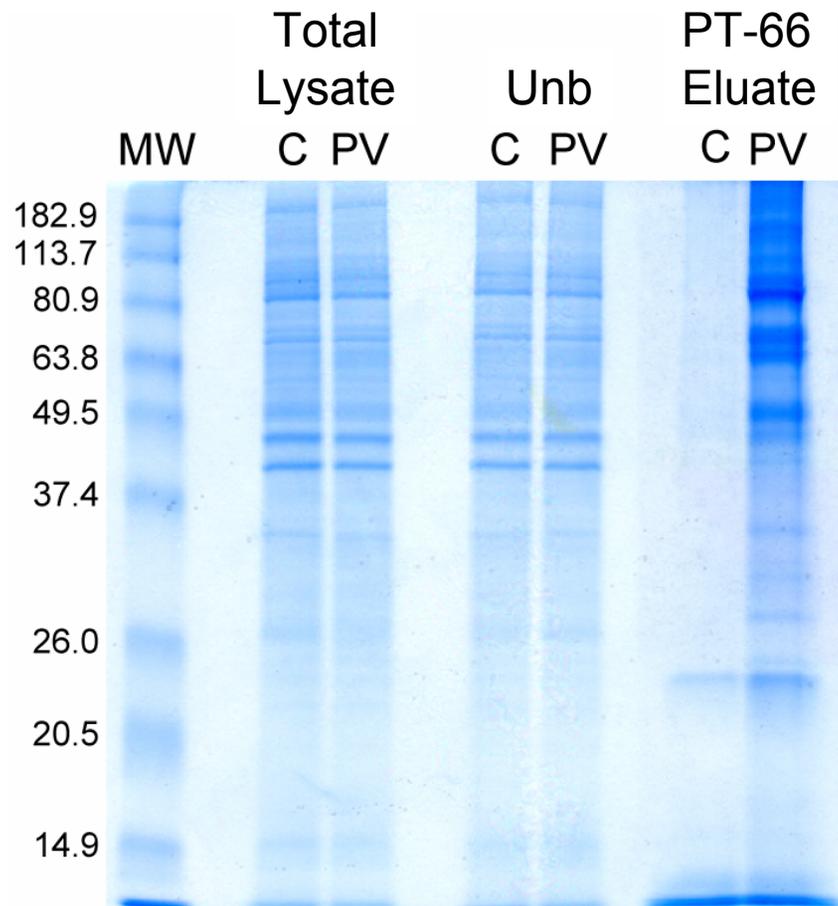
Generation of tyrosine-phosphorylated proteins by pervanadate treatment

To maximize detection and identification of phosphotyrosine-containing proteins, cells were treated with the tyrosine phosphatase inhibitor pervanadate. WEHI-231 cells were either left untreated (C) or treated with 100 μ M pervanadate for 2 hours (PV). The cells were lysed in RIPA buffer and proteins resolved on a 12.5% SDS gel. The proteins were either silver-stained or transferred to a nitrocellulose membrane and immunoblotted with anti-phosphotyrosine antibody PY99-HRP (Santa Cruz). The mass of each molecular weight marker (MW) is shown at the left.



Enrichment of tyrosine-phosphorylated proteins with anti-phosphotyrosine antibody PT-66

Lysates from control (C) or pervanadate-treated (PV) WEHI-231 cells were adsorbed to PT-66 agarose. After washing, the resin was eluted with triethylamine (TEA). Aliquots of the whole cell lysate (Total Lysate), the fraction not bound to PT-66 agarose (Unb), and the fraction eluted with TEA (Eluate) were resolved on 12.5% SDS gels and stained with Colloidal Blue. The mass of each molecular weight marker (MW) is shown at the left.

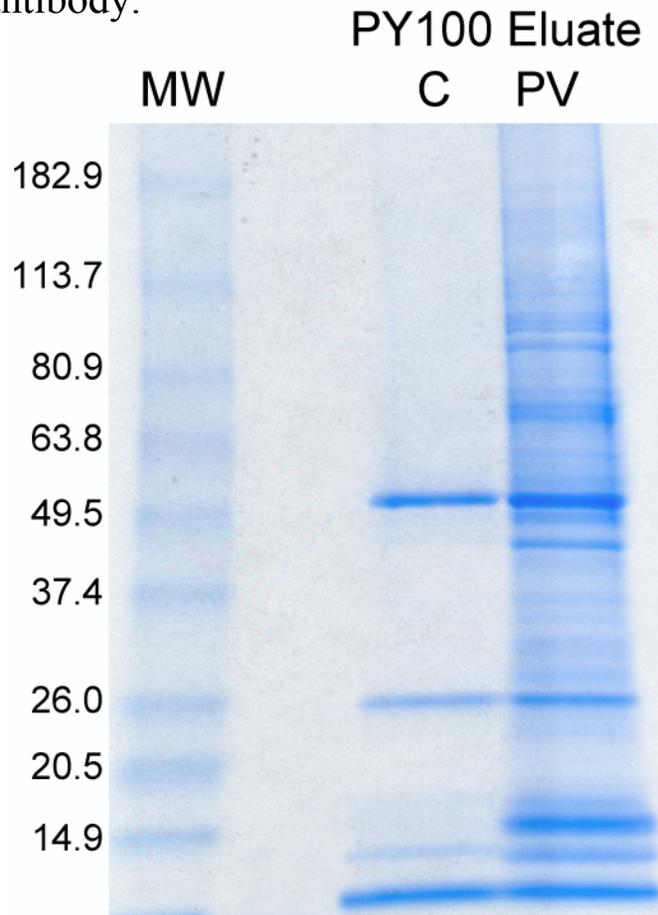


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Enrichment of tyrosine-phosphorylated proteins with anti-phosphotyrosine antibody PY100

Tyrosine-phosphorylated proteins were isolated from control (C) and pervanadate-stimulated (PV) WEHI-231 cells using PY100, as described for PT-66. Aliquots of the proteins eluted with TEA (Eluate) were resolved on a 4% to 20% gradient SDS gel and stained with Colloidal Blue. The mass of each molecular weight marker (MW) is shown on the left. The prominent bands migrating at 50 and 26 kDa are the heavy and light chains of the PY100 antibody.



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Identification of proteins enriched by immunoprecipitation with anti-phosphotyrosine antibodies

Table 1 (see link below) lists the proteins identified in four separate anti-phosphotyrosine immunoprecipitation experiments. A total of 120 proteins were identified, of which 33 were found in the AfCS Protein List. Twenty-nine of the identified proteins are known phosphotyrosine-containing proteins (PubMed links to relevant publications are included in the table). Nine previously uncharacterized novel proteins, predicted from genomic or cDNA sequences, were identified. The list also includes proteins that interact with tyrosine-phosphorylated proteins (e.g, SH2-containing proteins).

To control for proteins that might have bound nonspecifically to the antibody resins, samples from control and pervanadate-treated cells were compared. Regions from control lanes of the SDS gels were excised in parallel with the pervanadate treated-lanes. The gel pieces were digested with trypsin and analyzed by mass spectrometry. In general, no identifiable proteins were detected in the gel bands excised from the control lanes.

We encourage feedback to help us identify additional references reporting tyrosine phosphorylation of any of the proteins in this list.

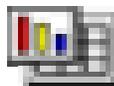


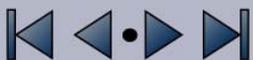
Table 1. *Proteins identified in anti-phosphotyrosine immunoprecipitates from pervanadate-treated WEHI-231 cells.*

Discussion

The major goal of the work reported here was the identification of tyrosine phosphorylated proteins in WEHI-231 cells. Identification of these proteins is part of the larger AfCS effort to map signaling pathways in B cells. This information will be used to better define the signaling networks present in these cells and provide new probes (primarily phosphospecific antibodies) for quantitative measurements of signaling molecule activation during ligand-induced signaling.

A total of 120 proteins were identified following immunoprecipitation with anti-phosphotyrosine antibodies. The proteins included known tyrosine-phosphorylated proteins and proteins important in B-cell signaling. However, Table 1 is not a complete list of tyrosine phosphorylated proteins in these cells. A number of B cell proteins known to be phosphorylated on tyrosine were not identified in these experiments (e.g., $Ig\alpha/\beta$, BLNK, Fyn, Blk, and Lyn).

The identified proteins were only present in immunoprecipitates from pervanadate-treated cells. This result indicates they were either phosphorylated on tyrosine or formed complexes with tyrosine-phosphorylated proteins. In order to differentiate these classes of proteins, we have begun to identify those proteins that contain phosphotyrosine. For example, a phosphorylation site was identified in valosin-containing protein by nanospray tandem mass spectrometry. The site identified (Tyr-806) corresponded to a previously identified site (4). We are currently using these methods to identify tyrosine-phosphorylated proteins and their phosphorylation sites in cells treated with physiological ligands.

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