Simple immunoprecipitation of phosphorylated proteins from pervanadate stimulated CHO cells using paramagnetic beads

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Simple immunoprecipitation of phosphorylated proteins from pervanadate stimulated CHO cells using paramagnetic beads

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Background

Fundamental cellular functions such as cell signalling are commonly regulated by phosphorylation of tyrosine residues in proteins (pTyr). When dysregulated they often play a prominent role in human cancer making the development of methods studying and identifying tyrosine phosphorylated proteins extremely important.

pTyr proteins are comparatively rare and difficult to measure. Threonine and serine phosphorylation constitute of 10 respectively 90 % of the total human phosphorylation, while the tyrosine phosphorylation only represent 0.05 %.

Phosphorylation of tyrosine residues in proteins is due to the activity of both tyrosine kinases and phosphotyrosine phosphatases. Specific phosphatase inhibitors such as vanadate and pervanadate are therefore useful tools in the study of tyrosine phosphorylation.

We have studied the effect of tyrosine phosphorylation of proteins in CHO cells after treatment with pervanadate, (generated by vanadate peroxidation in the presence of H_2O_2). A preparation method using immunoprecipitation with anti-phosphotyrosine (anti-pTyr) antibodies, cross-linked to Protein G Mag Sepharose™ magnetic beads (GEHC, Sweden) followed by nanoLC MS/MS mass spectrometry analysis has been developed.

By using a small amount of magnetic beads with highly selective antibodies, both enrichment and concentration of phosphorylated proteins is achieved resulting in an increased sensitivity and efficiency during MS analysis (fig. 1).

The binding capacity of the magnetic beads has been adjusted to reach a balance between the capacity and the amount of antibodies needed for a typical Immunoprecipitation experiment.

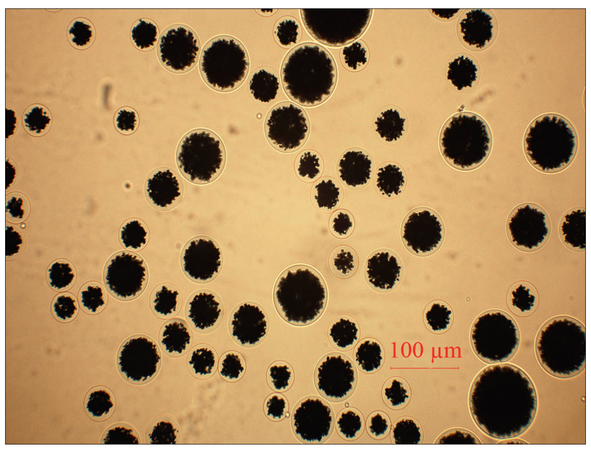


Fig 1. Microscopy picture of Mag Sepharose beads. The magnetite is centered in the middle of the particles with a thin shell of pure agarose on the surface.

Experimental overview

Proteins active in signaling pathways are normally not detectable with SDS-PAGE or MS analysis due to their low abundance. They are therefore requiring highly selective enrichments methods to be detected.

In combination with anti-pTyr antibodies, the Mag Sepharose technology is a powerful tool for capturing low abundant proteins involved in different signaling pathways from large amount of starting samples (fig. 2).

Finding low abundant tyrosine phosphorylated proteins in complex mixtures

CHO-cells (7×10^7) grown in rich medium were used as a source of tyrosine phosphorylated proteins. Pervanadate, a phosphatase inhibitor, was added two hours prior to harvest in order to prevent dephosphorylation. In a control experiment, this step was omitted.

The cells were lysed in mammalian protein extraction buffer supplemented with EDTA, Na-deoxycholat, protease inhibitors and phosphatase inhibitors. The lysate was clarified by centrifugation and diluted two-fold with binding buffer (TBS) before addition to Protein G Mag Sepharose. The pTyr proteins were eluted with 100 mM phenylphosphate (2 × 5 minutes in 37˚C). The eluates were digested with trypsin before analysis by LC-MS/MS.

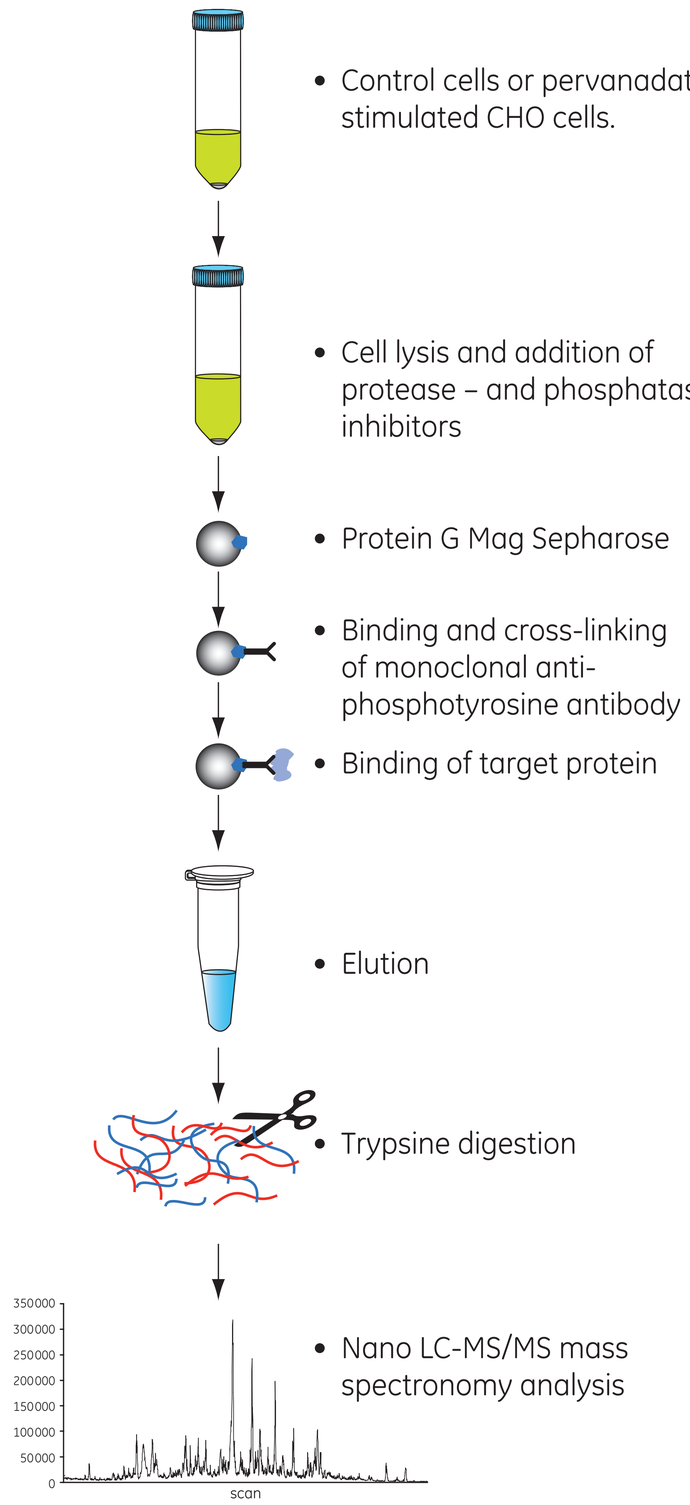


Fig 2. Workflow over the enrichment of tyrosine phosphorylated proteins from CHO cells using Protein G Mag Sepharose. A simple way of capturing low abundant proteins from small or large sample volumes.

Medium	100 µl Protein G Mag Sepharose 20 % gel slurry
Sample	CHO cells pre treated with pervanadate vs untreated CHO cells
Sample volume	10 ml
Capturing antibody	Monoclonal anti-phosphotyrosine antibody, clone PY20, isotype IgG2b
Binding buffer	Tris buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.5)
Wash buffer	TBS, 2 M urea, pH 7.5
Elution buffer	100 µl 100 mM phenylphosphate, pH 8

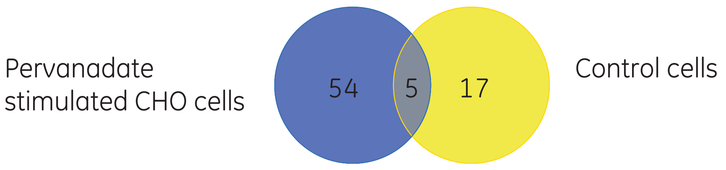
In the eluate, 76 potential tyrosine phosphorylated proteins were identified. Of these hits, 54 were exclusively found in the pervanadate treated cells and were neither found in the control cells nor in the start material (fig. 3 and table 1).

Some of these proteins such as Caveolin-1, FAK, and SHC are known to be involved in focal adhesion pathways. These proteins may be involved in the regulation of the actin cytoskeleton. Other proteins that are involved in cell motility or cell survival were also found. In the control samples (untreated cells), only 22 proteins were detected, mainly high abundant enzymes and ribosomal proteins. This example clearly demonstrates a simple and efficient capture of pTyr proteins.

Conclusions

- Efficient concentration and enrichment of tyrosine phosphorylated proteins from mammalian cells.
- The Mag Sepharose technology allows capture of low abundant proteins from large amount of starting samples and elution in MS suitable volumes.

Identified tyrosine phosphorylated proteins



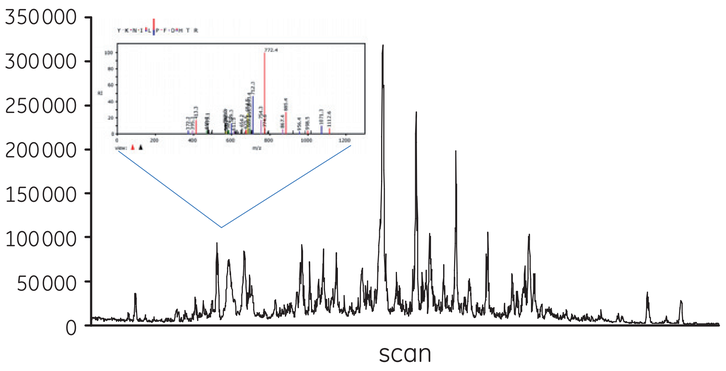


Fig 3. MS analysis of eluted proteins after immunoprecipitation of pTyr proteins from pervanadate-stimulated cells. Before analysis the proteins were cleaved with trypsin. An example MS/MS spectrum of a pTyr peptide is shown and was identified as PTPn11.

Table 1. The 20 most significant identifications exclusively found in the pervanadate stimulated CHO cells.

Protein	Total	M_r	Accession number
protein tyrosine phosphatase	22	68.3	gi|458333|
caveolin-1	11	20.5	gi|603661|
beta-tubulin isotype I [Cricetulus griseus]	6	49.6	gi|473884|
Cav1 protein [Rattus norvegicus]	1	19.7	(H) gi|124504347|
AHNAK [Mus musculus]	6	224.0	gi|37675525|
focal adhesion kinase	8	119.1	gi|193224|
beta tubulin [Cricetulus griseus]	1	49.7	(H) gi|537407|
gamma-actin	1	41.8	(H) gi|309089|
M1 pyruvate kinase [Rattus norvegicus]	5	57.8	gi|206204|
cortactin	5	61.2	gi|509495|
47-kDa heat shock protein [Mus musculus]	4	46.5	gi|303678|
ABL2 [Mus musculus]	3	128.1	gi|68139002|
SNAG1 [Mus musculus]	4	67.9	gi|15559064|
polymerase I-transcript release factor; PTRF [Mus musculus]	3	43.9	gi|2674195|
calmodulin synthesis	3	16.8	gi|192365|
beta-actin [Marmota monax]	1	32.0	(H) gi|9864780|
unnamed protein product [Rattus norvegicus], enolase 1	3	47.1	gi|56107|
eps8 binding protein [Rattus norvegicus]	3	51.7	gi|5882255|
Chain B, Refined 1.8 Angstroms Resolution Crystal Structure Of Porcine Epsilon-Trypsin	3	8.8	gi|999627|
Rous sarcoma oncogene [Mus musculus]	3	59.9	gi|123219085|

imagination at work

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