Bio-orthogonal Affinity Purification of Direct Kinase Substrates

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Protein kinases mediate signal transduction through phosphorylation of their protein substrates. Up to one-third of proteins in a cell are phosphorylated, and a major goal of phosphoproteomics is to characterize phosphorylation mediated signaling cascades by identifying phosphorylated proteins. This feat is analytically challenging because most phosphoproteins are of low abundance and substoichiometrically phosphorylated. Further, once a phosphoprotein is identified, it is difficult to integrate the role of the phosphorylation event into signal transduction networks without knowledge of the upstream kinase. Affinity purification techniques, such as strong cation exchange (SCX), immobilized metal ion affinity chromatography (IMAC), chemical tagging of phosphorylated residues with biotin, and phospho-motif specific antibodies can enrich phosphopeptides or proteins, but the information regarding the kinase responsible for phosphate transfer is uncoupled from the phosphorylation event. We previously have described methodology that allows selective labeling of direct kinase substrates, using analogue specific (as) kinases and orthogonal unnatural nucleotides (A*TP and A*TPyS); however, it remains challenging to biochemically isolate the labeled substrates, impeding their identification.

Here, we report a technique that combines direct substrate labeling with immunoaffinity purification (Schemes 1 and 2). To label the substrates of a given kinase, an as allele is used to enzymatically label substrates with A*TPyS. The selectively introduced thio phosphate is then chemically derivatized to construct a bio-orthogonal affinity tag. This approach is similar to other bio-orthogonal tagging strategies using ketones or azides, except thiophosphate cannot be selectively tagged in a single chemical step. For example, an alkylating agent will label both thio phosphate and other cellular nucleophiles, but we envisioned that an antibody could discriminate the thio phosphate alkylation products from other undesired alkylation products. The alkylating agent p-nitrobenzylmesylate (PNBM) was selected to construct the epitope because we predicted antibodies could recognize the product of thio phosphate alkylation over other nitrobenzyl alkylated amino acid residues, based on unique size and charge. Also, several high affinity antibodies have been raised against haptons containing p-nitrophenyl moieties, increasing the chances of eliciting an antibody capable of immunoprecipitation. Antibodies raised against hapten 4 are likely to be sequence independent because the binding determinants are relatively distant from the peptide backbone.

Utilizing hapten 4, polyclonal antibodies (IgY and IgG) were raised in chickens and rabbits, respectively. To enrich for specific binders, immune antibodies were purified on an affinity column containing immobilized hapten 4. Chicken IgY antibodies (α-3-IgY) performed best in immunoprecipitations and were used in subsequent experiments. To investigate α-3-IgY binding require-
immobilized on sepharose beads, were incubated with the rhodamine-labeled proteins, washed extensively, and the bound proteins were incubated with the rhodamine-

Figure 1. Recognition determinants for α-3-IgY immunoreactivity measured by western blotting; 25 ng of Swe1 or Swe1-P\(^{S}\) was treated with DMSO (lanes 1 and 3) or 2.5 mM PNBM in DMSO (lanes 2 and 4); 15 μg of WCL was treated with DMSO (lane 5), 2.5 mM PNBM in DMSO (lane 6), and 25 ng of Swe1-P\(^{S}\) plus 2.5 mM PNBM in DMSO (lane 7). Lanes 8 and 9 show coomassie staining of samples identical to 6 and 7, respectively.

Figure 2. Immunoprecipitation of Rh-H1-P\(^{S}\)+PNBM measured by fluorescence of the SDS–PAGE resolved immunoprecipitates. WCL\(^{*}\) indicates treatment with PNBM. Lanes 1–3 were treated with α-3-IgY sepharose, and lanes 4–5 with preimmune IgY sepharose.

strates from whole cell lysates will likely require production of anti-3 specific monoclonal antibodies, which is currently underway. The method we report here requires a kinase to utilize ATP\(^{S}\) as a phosphodonor, and although it is unclear what percentage of the kinase can utilize ATP\(^{S}\), several kinases have been shown to thio phosphorylate their substrates (see Supporting Information Table S5 for a partial list). Combining this purification method with as kinase substrate labeling should provide a general route to the identification of direct kinase substrates. Other biological questions may be approached with tandem chemical/immunological strategies,\(^{10}\) which are providing new routes to interrogate the proteome.

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**Supporting Information Available:** Details for experimental procedures, synthesis of hapten 4, ELISA data, Mob1 Western Blot, immunoprecipitation controls, and a table of ATP\(^{S}\) utilizing kinases. This material is available free of charge via the Internet at http://pubs.acs.org.

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**References**

(12) Mouse immune responses were weak (unpublished data).