Benchmarks

Isopropyl- β -D-thiogalactosidase (IPTG)-Inducible Tyrosine Phosphorylation of Proteins in *E. coli*

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The post-translational modification of proteins by tyrosine phosphorylation plays a critical role in signal transduction. As a consequence, this modification can dramatically alter the protein's activity or stability. To study the biological and biochemical properties of these proteins in vitro, a source for obtaining an adequate amount of phosphorylated protein is essential. In many instances, the phosphoprotein is expressed in cell systems such as baculovirus/insect cells or eucaryotic cell lines expressing an appropriate kinase. However, the use of these methods is relatively time consuming. Alternatively, the recombinant proteins can be phosphorylated in vitro with purified kinase, but the cost associated with these procedures is often prohibitive.

Phosphorylation of mammalian proteins expressed in bacteria has been described (2,3,7). In a commercially available system, Epicurian Coli® TKX1 Competent cells (Stratagene, La Jolla, CA, USA), a mammalian tyrosine kinase encoding plasmid under the control of the procaryotic *trp* promoter is used to phosphorylate target proteins expressed in *E. coli*. If the target protein has an affinity tag or is fused to glutathione-S-transferase (GST) or maltose binding protein (MBP), then the production and purification of large amount of this protein is relatively rapid and easy. However, the use of the *trp* promoter is rather cumbersome. To induce the expression of the kinase, the cells must be transferred to M9 minimal medium and treated with indole acrylic acid. To make this method of generating phosphoproteins more "user friendly", we generated a plasmid that controls the expression of the tyrosine kinase with the hybrid *tac* promoter (4). This plasmid, pTac-elk, encodes a rat tyrosine kinase (elk) (5) whose expression is induced by treatment with isopropyl- β -D-thiogalactosidase (IPTG).

The plasmid also encodes tetracycline resistance, making it easy to co-select with many other conventional ampicillin-resistant vectors.

The plasmid harbored in the *E. coli* strain TKX1 (Stratagene) was isolated. The *trp* promoter was replaced by *tac* promoter from pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by a two-step PCR method (shown schematically in Figure 1). In brief, a pair of primers was designed containing an initiation ATG in the middle and homology to the *tac* promoter sequence of pGEX2T upstream and homology to elk coding region downstream (plus-tac: 5'-ttcacacagaaacagatttcATGGAAGCTGTCCGGGAGTTTGCC-3'; minus-tac: 5'-GGCAAAC-CCCGGACAGCTTCATgaatactgttcctgtgtgaa-3'; the lowercase letters indicate the sequence from pGEX2T, and the uppercase letters indicate the sequence from elk). The PCR product containing *lac* operator and *tac* promoter from pGEX2T were generated by primer up-tac (5'-gcgcccatggcatcatacggttctgac-3') and minus-tac, and a *Nco*I site (underlined) was added to its 5' end.

The second PCR product containing the elk segment was amplified with primer down-elk (5'-ctttgcacaccaggttgc-3') and plus-tac. The resulting two PCR products were purified, mixed together along with the flanking primers up-tac and down-elk, and reamplified. This PCR product was restricted with *Nco*I and *Apa*LI and gel purified. This fragment was used to replace the *Nco*I and *Apa*LI fragment of the progenitor plasmid (containing the *trp* promoter). The integrity of the resulting plasmid was confirmed by DNA sequencing.

To test the induction of the pTac-elk plasmid, a fragment of Stat3 α (amino acids 570-770), containing tyrosine 705, was amplified by PCR and cloned into pGEX2T. JM 109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]*) were made competent using ice-cold 50 mM CaCl₂ and transformed with pTac-elk, and tetracycline-resistant colonies were selected. The resulting cells were then made competent and transformed with the Stat3-GST plasmid. Double-resistant (Amp and Tet) colonies were picked and cul-

Figure 1. Schematic representation of generating pTac-elk. PCR product 1 was generated using pGEX-2T as a template and primers up-tac and minus-elk. PCR product 2 was generated using the *trp*-elk plasmid (Stratagene) and primers plus-tac and down-elk. The resulting products were purified, mixed, and reamplified with primers up-tac and down-elk. This product was digested with *Nco*I and *Apa*LI and used to replace the *Nco*I-*Apa*LI fragment of the *trp*-elk plasmid (containing the *trp* promoter).

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tured in 2 mL LB media. The next day, this culture was used to inoculate larger cultures at 1:50 dilution in Terrific Broth (6). After 3 h of incubation, 1 mM IPTG was added to the culture medium. After an additional 2 h of incubation, the cells were harvested. Cell pellets were resuspended and boiled in 1×SDS loading buffer, and cell equivalents were electrophoresed on SDS-acrylamide gels. The separated proteins were then analyzed by immunoblotting with phospho-Y-705 Stat3 antibody (Cell Signaling, Beverly, MA, USA).

As seen in Figure 2, IPTG-inducible phosphorylation of the GST-Stat3 fusion protein was observed. Since the antibody employed specifically recognizes phospho-Y-705, this indicated that the fusion protein was specifically phosphorylated on tyrosine-705. Furthermore, comparison of the signal before and after IPTG induction strongly indicates that the expression level of the elk kinase was controlled by the *tac* promoter (the faint band in the uninduced lane indicates that the *lacI^q* control of the *lac* and *tac* promoters is slightly leaky). We have found that cells grown from different colonies from the same plate result in different magnitudes of induction. Therefore, we suggest growing several independent clones from each transformation to find those with maximal induction of protein phosphorylation.

The primary advantage of this method over the existing system is the

improved convenience of producing the target phosphoprotein. If the target protein is under control of the *lac* or *tac* promoter, then the expression of the target protein and kinase can be induced simultaneously. This is advantageous if the target protein is toxic to the host *E. coli* strain. Furthermore, there is no need to change the cells to minimal medium and induce with indole acrylic acid that also facilitates the production of phosphoprotein. We have successfully used these cells to purify several tyrosine phosphoproteins, including full-length Stat3 α and portions of the cytoplasmic domain of the receptor tyrosine kinase erbB2. We have detected inducible tyrosine phosphorylation with all proteins we have tested to date. Since pTac-elk plasmid does not contain *lacI^q*, tight control of the expression of the elk kinase requires the use of an *E. coli* strain that expresses *lac* repressor. Although the GST-Stat protein we presented here to demonstrate the usefulness of the system was rather small (only approximately 200 amino acids of the Stat3 protein), we have detected tyrosine phosphorylation of full length Stat3 α , which is approximately 92 kDa, indicating that larger proteins are also efficient substrates of the kinase. This system is designed to use the expression of a promiscuous tyrosine kinase to phosphorylate proteins expressed in bacteria. How specific is the kinase activity? We have shown using a specific antibody that the only known tyrosine

that is phosphorylated in mammalian cells is properly phosphorylated using these Elk-expressing cells. Another group, using the BL21(DE3)TBK1 cells (Stratagene) to phosphorylate Stat3 β , found that tyrosine-705 was the major phosphorylated residue following purification and liquid chromatography-mass spectrometric analysis LC-MS (1). We suspect that tyrosine residues on proteins other than those that are “natural” substrates of tyrosine kinases are also phosphorylated by expression of Elk. This is based on the fact that numerous bands are detected in extracts from IPTG-induced cells using the pan-phosphotyrosine specific antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, USA). This indicates that many endogenous *E. coli* proteins that normally do not undergo tyrosine phosphorylation are substrates for the Elk kinase. Based on this observation, the efficiency and specificity of the tyrosine phosphorylation of any target protein will have to be determined empirically.

In summary, the pTac-elk plasmid offers a more convenient means to generate mammalian tyrosine phosphoproteins in bacteria than is currently available. Since this post-translational modification is essential for the function of proteins in numerous signal transduction pathways, the use of this system will allow for the generation of phosphoproteins for in vitro studies such as protein-protein interactions or X-ray crystallography, in which post-translational modification by tyrosine phosphorylation is required.

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Figure 2. IPTG induction of Stat3-GST fusion protein by elk kinase. JM109 cells were transformed with pTac-elk and pGST-Stat3 α (amino acids 570–770) as described in the text. JM109 cells harboring both plasmids were grown in culture with selection until early log phase (approximately 3 h). The cells were then split into two equal volumes. One tube was induced with IPTG, while the other was left untreated. Cells were grown for an additional 2 h and collected by centrifugation at 5000× g. The cell pellets were resuspended in equal volumes of SDS buffer. (–) uninduced; (+) induced with IPTG. Equal volumes of cell extracts (representing equivalent numbers of cells) were separated by SDS-PAGE and immunoblotted with an antibody specific for the tyrosine-705 phosphorylated form of Stat3 α .

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