Western blot analysis of Src kinase assays using peptide substrates ligated to a carrier protein

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We have applied intein-mediated peptide ligation (IPL) to the use of peptide substrates for kinase assays and subsequent Western blot analysis. IPL allows for the efficient ligation of a synthetic peptide with an N-terminal cysteine residue to an intein-generated carrier protein containing a cysteine reactive C-terminal thioester through a native peptide bond. A distinct advantage of this procedure is that each carrier protein molecule ligates only one peptide, ensuring that the ligation product forms a sharp band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We demonstrate the effectiveness of this approach by mutational analysis of peptide substrates derived from human cyclin-dependent kinase, Cdc2, which contains a phosphorylation site of human c-Src protein tyrosine kinase.

INTRODUCTION

Protein phosphorylation plays a pivotal role in many signal transduction pathways (1,2). Yet the dissection of kinase signaling pathways presents a challenge to existing techniques (3). The use of synthetic peptide substrates has become a powerful tool to determine kinase specificities, allowing mutational analysis and investigation into optimal phosphorylation sites (4). One extensively used technique for kinase activity assessment is the measurement of the incorporation of radiolabeled γ-phosphate from ATP (5). An alternate approach for the detection of protein phosphorylation is to determine the immunoreactivity of a protein to a phosphor-specific antibody that recognizes the phosphorylated epitopes within proteins (6). Two common methods for the determination of immunoreactivity are enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Because of the small size of synthetic peptides, typically in the range of 10–20 amino acid residues, they are not suitable for Western immunoblotting analysis.

In this report, we describe an easy and effective method for producing carrier protein-peptide ligation products for kinase assays and subsequent Western blot analysis. A peptide possessing a phosphorylation site of interest is first synthesized with an amino-terminal cysteine residue. The peptide is then ligated to the cysteine-reactive carboxyl terminus of a carrier protein via a peptide bond by intein-mediated protein ligation (Figure 1) (7–9). This results in the ligation product migrating as a single band on a sodium dodecyl sulfate (SDS) polyacrylamide gel.

MATERIALS AND METHODS

Generation of Peptides and Carrier Protein

All peptides were synthesized with an N-terminal cysteine and purified by high-performance liquid chromatography (HPLC) (New England Biolabs, Beverly, MA, USA) (Table 1; References 10 and 11). For ligation to the peptides, the paramyosin ΔSal fragment from Dirofilaria immitis (12) was expressed as a paramyosin-intein-chitin binding domain fusion protein (PXB) and purified on chitin resin. Intein-mediated cleavage was carried out at 4°C for 16 h by incubation of the chitin resin in column buffer containing 20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, and 50 mM 2-mercaptoethanesulfonic acid (MESNA; Sigma, St. Louis, MO, USA). Protein elutions were collected in 5-mL fractions from the column, and protein concentrations were determined by Bradford assay (13).

Ligation of Carrier Protein and Peptide

For the experiment shown in Figure 2, the ligation reactions were performed overnight at 4°C in a 100-μL reaction volume with 500 μM of peptide and 20 μM of paramyosin carrier protein in the presence of 100 mM Tris-HCl, pH 8.5, and 10 mM MESNA. For the experiment shown in Figure 3, the ligation reactions were carried out as described above except for a shorter ligation time (4 h at 4°C) and an increase in peptide concentration to 1 mM final concentration.

Kinase Assays

The ligated samples were dialyzed against 5 mM Tris-HCl, pH 7.5, 50 mM NaCl to remove the unligated peptide using a 0.025 μm filter (Millipore, Bedford, MA, USA). Kinase assays were carried out at 30°C for 60 min in a 10-μL reaction with each sample containing 10 μM (or 0.30 mg/mL) of the carrier protein and 12.5 U Src kinase (Upstate, Lake Placid, NY, USA) in the presence of 100 μM ATP and 25 mM Tris-HCl, pH 7.2, 31.25 mM MgCl₂, 25 mM MnCl₂, 0.5 mM ethylene glycol-bis(2-}

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Western Blot Analysis

Western immunoblotting analysis was performed using an anti-phosphotyrosine antibody (P-Tyr-100; Cell Signaling Technology, Beverly, MA, USA). Each kinase assay sample was diluted 21-fold into an SDS-containing loading buffer. The samples were heated to 95°C for 5 min before an aliquot (10 μL) of each assay sample was loaded onto a 12% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). Proteins were resolved by electrophoresis in SDS-containing running buffer. Proteins were transferred to a 0.45 μm nitrocellulose membrane and then blocked with TBSTT (Tris-buffered saline, pH 7.5, 0.2% Tween20, 0.05% Triton® X-100) supplemented with 5% nonfat dry milk at 25°C for 1 h. The membrane was probed with the primary antibody (P-Tyr-100) prepared by 1:5000 dilution in TBSTT supplemented with 2% nonfat dry milk. After an overnight incubation at 4°C, the membrane was washed three times with TBSTT and incubated with a horseradish peroxidase (HRP)-linked anti-mouse immunoglobulin G (IgG) antibody (1:5000 dilution in TBSTT with 2% dry milk) at room temperature for 1 h, followed by three additional 15-min washes in TBSTT. Antibody binding was then detected by chemiluminescence with the Phototope®-HRP Western Blot Detection System (Cell Signaling Technology). The membrane was further incubated with a rabbit anti-paramyosin antibody (produced by Covance Research Products, Danver, PA, USA). Detection was performed with the Phototope-HRP Western Blot Detection System.

RESULTS AND DISCUSSION

We have designed a series of peptides that are derived from the substrates of human c-Src protein tyrosine kinase (Src-PTK) possessing an additional N-terminal cysteine residue (Table 1). The peptide Src-Y contains the optimal sequence predicted for the Src kinase (4). The Cdc2-Y15 peptide is a substrate for Src PTK corresponding to I10 to K20 of human cyclin-dependent kinase, Cdc2 (10). Mutational study indicates that phosphorylation at Tyr15 on Cdc2 has a regulatory role in the cell cycle (11). To evaluate whether this technique can be applied to the investigation of the effect of mutations surrounding a phosphorylation site, single amino acid substitutions were introduced at six different positions (Table 1). Also included were a phosphorylated peptide (Cdc2-pY15) and a negative control peptide (Cdc2-Y15F) with Tyr15 substituted with phenylalanine residue.

A 27-kDa carrier protein, paramyosin ΔSal fragment from D. immitis (12) was produced according to the method described previously (8). The paramyosin protein was expressed as a tripartite fusion protein consisting of the paramyosin protein segment, which is fused at its C terminus to the N terminus of the Mycobacterium xenopi GyrA intein (22 kDa), followed by a chitin-binding domain (CBD; 7 kDa) from Bacillus circulans. Intein-mediated cleavage was induced by incubation of chitin-bound fusion protein with MESNA, resulting in the release of paramyosin carrier protein possessing a carboxyl-terminal thioester group.

The carrier protein was ligated to six different substrates or control peptides. The ligation reactions were performed overnight at 4°C with 500 μM phosphate and 20 μM carrier protein. Proteins were resolved by electrophoresis on a 12% SDS polyacrylamide gel (Figure 2A). The covalent linkage of peptide to the carrier protein caused a mobility shift on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Since the stoichiometry of this ligation reaction is precisely
one-to-one, each paramyosin-peptide ligation product formed a single sharp band. The extent of ligation is indicated by the ratio of the ligated (or shifted band) and unligated paramyosin carrier proteins. Figure 2A shows that the lowest ligation efficiency, of approximately 50%, was observed for Cdc2-pY15 (lane 3) and Src-Y (lane 4) and is likely due to their poor solubility. The other Cdc2-derived peptides exhibited approximately 80%–90% ligation efficiency.

Next, the ligated samples were dialyzed to remove the unligated peptide. Kinase assays were carried out at 30°C for 60 min, with each sample containing 10 μM (or 0.30 mg/mL) of the carrier protein and 12.5 U Src kinase in the presence of 100 μM ATP. Western blot analysis was performed using an anti-phospho-tyrosine monoclonal antibody that recognizes peptides containing phosphotyrosine. Figure 2B reveals that Src-Y, Cdc2-Y15, and Cdc2-Y19F (lanes 2–4) peptides exhibited positive signals comparable to the control Cdc2-pY15 (lane 1), which contains a phosphorylated tyrosine residue. As expected, the mutation of Tyr15 in Cdc2-Y15F resulted in no detectable signal. Cdc2-G13K exhibited a significant reduction in signal, suggesting that this substitution...
significantly affected the efficiency of Src-mediated phosphorylation at Tyr15. The presence of a weak signal at 60 kDa in each lane is likely due to autophosphorylation of the Src kinase as previously reported (14). To detect any significant protein loading variations, the membrane was incubated with a rabbit anti-paramyosin antibody, following the first immunoblot analysis with the anti-phospho-tyrosine antibody. The results confirmed the presence of similar amounts of paramyosin carrier protein in all lanes (data not shown).

Furthermore, we sought to analyze the entire set of mutant peptide substrates (Table 1). The ligation reactions were performed according to the procedure described above, except for a shorter ligation time (4 h at 4°C) and an increase in peptide concentration to 1 mM final concentration. The ligation efficiencies were in the range of 70%–90% as evaluated by Coomassie® Blue-stained SDS-PAGE (Figure 3A). The kinase assays were performed directly using the ligation samples containing 250 μM peptide and 5 μM carrier protein. The assays were examined by Western blot analysis reacted with the anti-phospho-tyrosine antibody (Figure 3B). The data suggests that mutation of the amino acid residues in proximity to Tyr15 as shown in the T14K or G16F substitution almost completely blocked phosphorylation by Src kinase, whereas the substitution of E12, G13, and V18 had significant but reduced effects compared to the T14K and G16F substrates. In addition, autophosphorylation of Src kinase was detected by the presence of a signal at 60 kDa in all the samples including the control with Src kinase alone (in the absence of the substrate). Finally, the Western blot was reacted with the rabbit anti-paramyosin antibody to verify the approximate quantity of carrier proteins in each lane (Figure 3C).

In conclusion, we have developed a sensitive method for kinase assays by ligating a peptide substrate via its N-terminal cysteine residue to the C terminus of a paramyosin carrier protein. This approach allows for the examination of kinase activity using small synthetic peptide substrates by Western immunoblotting analysis. The procedure is convenient and involves only minimum chemical manipulation (with MESNA), which permits use in most laboratories. Thioester-tagged carrier proteins of different sizes can be readily purified using commercially available intein vectors (7,8). The ligation efficiency can be evaluated based on a mobility shift on SDS-PAGE. Since the ligation is usually conducted with a 25- to 50-fold excess of synthetic peptide, the purity of a peptide sample does not significantly influence the yield of the ligation product. One significant advantage is that this method does not depend on the use of radiolabeled ATP or the addition of basic residues to the peptide, as required for binding to phosphocellulose paper (5). The carrier-peptide substrates can be utilized for semiquantitative analysis (6). Furthermore, the signal from kinase autophosphorylation is distinguishable from that of a substrate as long as a carrier of different size is chosen. Recently, we have conducted the assay of a peptide substrate of Abl protein tyrosine kinase using paramyosin as the carrier (data not shown). Thus, the method presented may be applied to other enzymatic assays for the study of protein modification. However, this technique relies on the availability of highly specific antibodies, such as anti-phospho-tyrosine antibody. This approach can yield direct evidence of kinase and cognate substrates and has the potential utility in the screening of optimal sequences.

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