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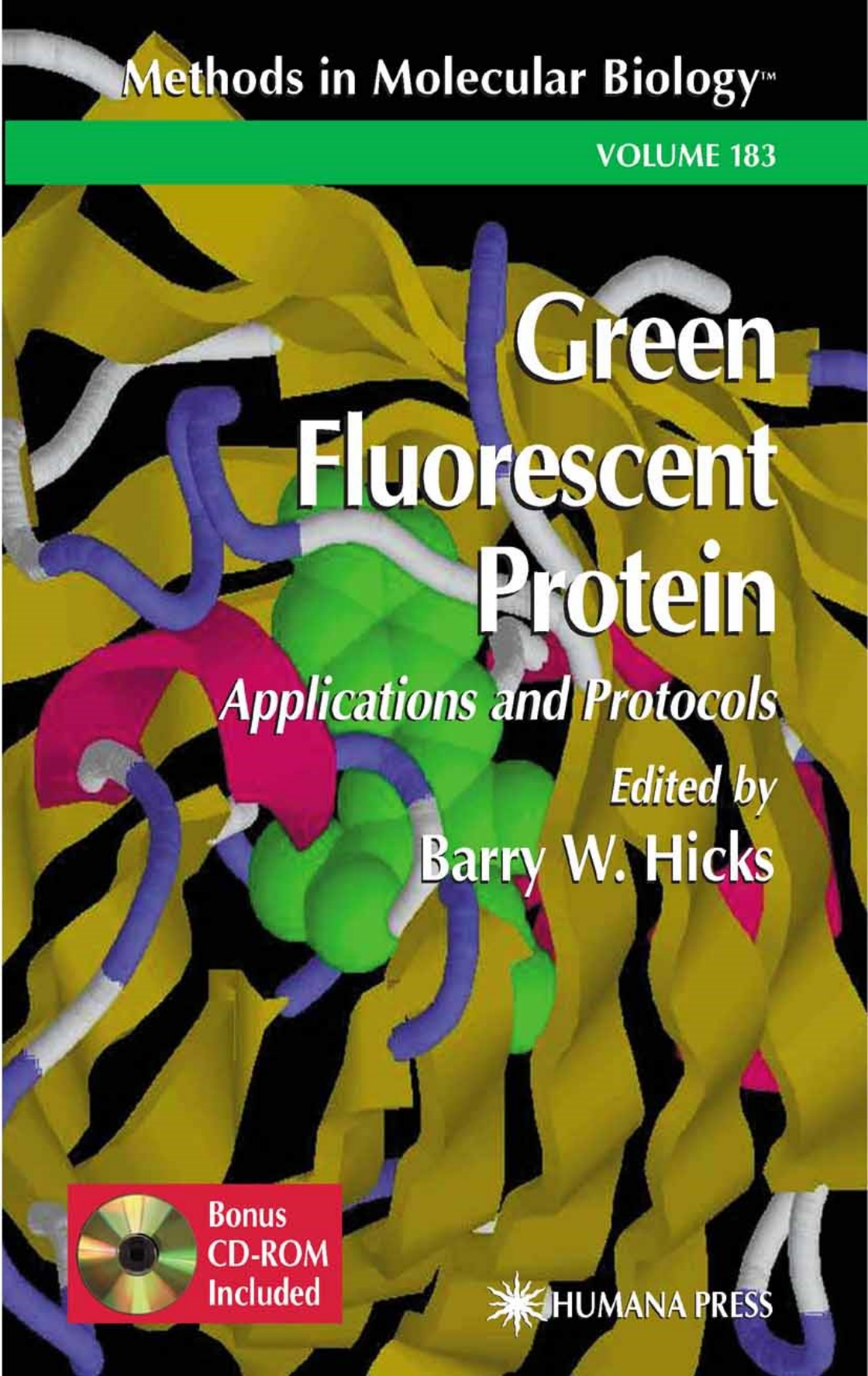

Green Fluorescent Protein

Applications and Protocols

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Green Fluorescent Protein-Based Protein Kinase Biosensor Substrates

Scott Ulrich and Kevan Shokat

1. Introduction

Protein kinases (PKs) are a family of enzymes that catalyze the transfer of γ-phosphate from adenosine triphosphate (ATP) to tyrosine, serine, or threonine amino acid residues of substrate proteins. Phosphorylation alters the enzymatic activity, binding capability, or cellular localization of the substrate protein, as a means to relay environmental signals, such as the extracellular matrix, antigens, insulin, and growth factors ***(1)***. Following the discovery of protein phosphorylation as a mechanism of signal transduction, the discovery of the v-*src* and v-*abl* oncogenes ***(2,3)***, and the realization that PKs are an immense superfamily of proteins (2.1% of *Caenor elegans* genes are PKs); PKs have moved to center stage in the field of signal transduction. Because of their centrality in cell signaling, PKs have also become attractive therapeutic targets for such diverse diseases as diabetes and cancer ***(4)***.

Biochemical study of PKs necessitates robust in vitro methods to assay kinase activity of recombinant and immunoprecipitated kinases. Often, exogenous peptide or protein substrates are added as phosphoacceptors in these assays. Random polypeptides such as poly Glu:Tyr (4:1) have been used in high-throughput kinase assays ***(5–7)***. Peptide substrates can be optimized by screening a combinatorial peptide library for efficient phosphorylation sequences, yielding highly efficient substrates in a kinase-specific manner ***(8–15)***. Kinase activity can then be assayed by quantitating transfer of ^{32}P to the peptide. However, peptides cannot be used in gel-based assays such as Western blotting. The protein substrates used for this purpose are often fortuitous, nonoptimal proteins such as histone H1, enolase, and immunoglobulin

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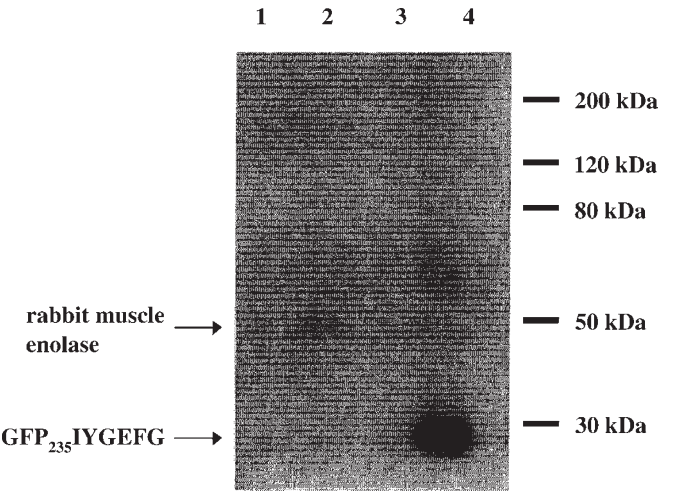

Fig. 1. Comparison of GFP, enolase, and GFP_{235}IYGEFG as XD4 substrates in a radioactive assay. Lane 1, 1 µg GFP; lane 2, 5 µg activated enolase; lane 3, 5 µg enolase; lane 4, 0.5 µg GFP_{235}IYGEFG. Reactions were run as described under Materials and Methods. Samples were run on a 12% SDS-PAGE gel. The gel was dried and exposed to an X-ray film at room temperature. Exposure time was 24 h.

heavy chain. Recognizing the need for efficient protein substrates for in vitro kinase reactions, our lab has developed a general method to transfer the advantages of a combinatorial peptide optimization strategy to the development of efficient green fluorescent protein (GFP), (this chapter refers to GFPMut1 ***[16]*** as GFP)-based PK substrates suitable for protein-based assays.

GFP is a compact protein that is easily expressed in prokaryotes and is very poorly phosphorylated by PKs (**Fig. 1**). The strategy used to transform GFP into an efficient kinase substrate involves starting with the optimal phosphorylation sequence of a given kinase gained through phosphorylation of a peptide library. The optimal phosphorylation sequences of many well-studied kinases are known ***(8–15)***. This efficient phosphorylation sequence is then appended to the C-terminus of GFP as a flexible tail. This laboratory has demonstrated that these modified GFP proteins are efficient protein substrates that can be easily generated in a kinase-specific manner. The resulting GFP constructs are easy to express, easy to purify using standard 6× His affinity tags, and easy to use in protein-based kinase assays. The GFP constructs shown in **Table 1** retain the fluorescence and expression qualities of wild-type GFP (data not shown). The suitability of GFP as a carrier protein for an appended phosphorylation sequence is also verified by its lack of endogenous phosphorylation sites, which, if present, would complicate antiphosphotyrosine immunoblotting

Table 1
GFP Constructs

Kinase	GFP Sequence-Optimal Phosphorylation Sequence	Name of Construct
	^{215}RDHMVLLEFVTAAGITHGMDELYK^{238}	GFP
Src	RDHMVLLEFVTAAGITHGMDEIYGEFGGS	GFP_{235}IYGEFG
Src	RDHMVLLEFVTAAEIYGEFG	GFP_{227}EIYGEFG
Abl	RDHMVLLEFVTAAGITHGMDAIYAAPF	GFP_{235}AIYAAPF
PKA	RDHMVLLEFVTAAGITHGMDRRRRSII	GFP_{234}RRRRSII
PKA	RDHMVLLEFVTAARRRRSII	GFP_{227}RRRRSII
PKA	RDHMVLLEFVTAARRSII	GFP_{227}RRSII

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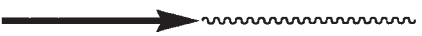

(**Fig. 1**, lane 1). GFP is then purified from its prokaryotic expression host as an unphosphorylated, well-expressed, fluorescent protein. These constructs have proven to be much more efficient phosphoacceptors than proteins commonly used as kinase substrates. Serine/threonine kinases and tyrosine kinases have both had phosphorylatable GFP constructs made and the method appears to be general and useful to rapidly generate sensitive in vitro substrates for a variety of PKs.

In searching for a versatile, nonradioactive kinase assay system, the authors sought to combine the advantages of highly efficient peptide substrate sequences with the convenience of protein substrates useful in polyacrylamide gel electrophoresis (PAGE)-based kinase assays. GFP was chosen as a protein host for optimal substrate sequences derived from combinatorial peptide libraries. Its small size (28 kDa), ensures that it will separate easily from most, if not all, protein components isolated in immune complex kinase assays (majority >35 kDa). GFP is a highly expressed protein that is easy to purify and handle. By combining the efficiency of optimal phosphorylation sequences with these qualities, C-terminal tagged GFP constructs have been shown to be excellent substrates for a variety of PKs.

2. Materials

1. DNA plasmids: Plasmid pGFPMut1, which contains the *GFPMut1* gene in a pKEN vector, was a gift from B. P. Cormack (Stanford University). Plasmid vector pQE8 is from Qiagen (Chatsworth, CA). pGEX-KT-XD4 and the c-Ab1 expression vector have been described ***(16,17)***.
2. Qiaex II kit were purchased from Qiagen.
3. Restriction enzymes: *Bam*HI.

4. Calf intestinal alkaline phosphatase (CIP), and T4 DNA ligase (New England Biolabs, Beverly, MA).
5. Luria-Bertoni (LB) broth and LB agar (Bio101, La Jolla, CA).
6. *Escherichia coli* strain JM109.
7. Isopropyl β-D-thiogalactoside.
8. Protein kinase A (PKA) (Life Technology, Gaithersburg, MD).
9. Talon resin (Clontech, Palo Alto, CA).
10. [γ-^{32}P]ATP (Du Pont NEN, Boston, MA).
11. Antiphosphotyrosine monoclonal antibody 4G10 was a gift from Brian Druker (Oregon Health Science Center, Portland, OR).
12. SuperSignal chemiluminescent substrate for horseradish peroxidase (Pierce, Rockford, IL).
13. *Pfu* polymerase, deoxyribonucleoside triphosphate (dNTPs), and polymerase buffer (Stratagene, La Jolla, CA).
14. 1% Agarose gels.
15. 12% Sodium dodecyl sulfate (SDS)-PAGE gels.
16. Nitrocellulose transfer membrane.
17. Electrospray mass spectrometry was performed by the Mass Spectrometry Facility, Department of Chemistry, Princeton University, with a Hewlett-Packard 5989B spectrometer.
18. Oligonucleotide synthesis and automatic DNA sequencing were done in the Synthesis and Sequencing Facility at Princeton University. Polymerase chain reaction (PCR) reverse primers used in this work are listed below. The forward primer for all constructs was 5'-TCTAGGGATCCGGCATGAGTAAAGGA-3'.
    a. pGFP$_{235}$IYGEFG
    5'-TCTAGGATCCGCCGAATTCGCCGTATATTTCATCCATGCCATG-3'.
    The resulting construct replaces the last three codons of GFP with **IYGEFGGS.**
    b. pGFP$_{227}$EIYGEFG
    5'-TCTAGGATCCTTAGCCGAATTCGCCGTATATTTCAGCAGCTGT-TACAAACTCAA-3'.
    The resulting construct replaces the last 11 codons of GFP with **IYGEFG.**
    c. pGFP$_{235}$AIYAAPF
    5'-ACGTATTCGAATTAGAACGGCGCCGCATAGATCGCTTCATC-CATGCCATGTGTAATC- 3'.
    The resulting construct replaces the last three codons of GFP with **AIYAAPF.**
    d. pGFP$_{234}$RRRRSII
    RP, 5'-GATAGGATCCTTAGATGATAGATCTAGGCCGGCGATCCATG-CCATGTGTAATC-3'.
    The resulting construct replaces the last four codons of GFP with **RRRRSII**.
    e. pGFP$_{227}$RRRRSII
    5'-TCTAGGATCCTTAGATGATAGATCTACGCCGGCGAGCAGCTGT-TACAAACTCAA-3'.
    The resulting construct replaces the last 11 codons of GFP with **RRRRSII**.

f. $pGFP_{227}RRSII$
The last 11 codons of GFP are replaced with **RRSII,** the serine residue of which was at the same distance from the GFP core structure as that of tyrosine in $GFP_{227}EIYGEFG$.

19. Src buffer: 100 m*M* HEPES, pH 7.4, 10 m*M* $MgCl_2$, 10 µg/mL bovine serum albumin, 0.1 m*M* dithiothreitol, 1 m*M* sodium orthovanadate, 0.2 m*M* ATP.
20. c-Ab1 buffer: 50 m*M* Tris-HCl, pH 8.1, 10 m*M* $MgCl_2$, 200 m*M* ATP.
21. PKA (Sigma).
22. PKA buffer: 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 20 mM ATP.
23. Tris buffer: 20 m*M* Tris-HCl, pH 8.0.
24. Elution buffer: Tris buffer plus 200 m*M* imidazole.
25. 5% Dried milk in phosphate-buffered saline.

3. Methods

3.1. Procedures for Plasmid Construction (see Note 1)

1. Using the PCR, add a *Bam*HI site to the end of *GFPMut1* gene in pGFPMut1.
2. Add a nucleotide sequence coding for the peptide of choice, followed by a *Bam*HI site to the 5' end of the gene, corresponding to the C-terminus of the GFP.
3. Perform PCR using *Pfu* polymerase and dNTPs, according to manufacturer's instructions.
4. Digest the PCR product with *Bam*HI.
5. Separate the nucleic acid digest by agarose gel electrophoresis, and extract the appropriate fragment, using the Qiaex II kit.
6. Similarly, digest the vector, pQE8, which contains a 6X His coding sequence before a *Bam*HI site, treat with CIP.
7. Separate the pQE8 digest by agarose gel electrophoresis, and extract the appropriate fragment.
8. Ligate the 6X His vector and the GFP-kinase sensor insert using T4 DNA ligase according to manufacturer's instructions.
9. Use the ligation mixture to transform competent JM109 *E. coli*. Screen colonies using a hand-held ultraviolet lamp, and pick green fluorescent colonies.
10. Isolate plasmid DNA from these colonies and analyze by restriction digest. Confirm positive clones by sequencing.

3.2. Expression and Purification of GFP Kinase Sensors

1. Grow the transformed *E. coli* harboring one of the constructs overnight at 37°C in LB broth with 75 µg/mL ampicillin. Dilute the culture 1:100 into 50 mL of same medium, and grow at 37°C until OD_{596} reaches 0.4 (~3 h).
2. Add IPTG to a final concentration of 0.5 m*M*, and grow the culture for an additional 8–10 h.
3. Harvest the cells by centrifugation and lyse them by sonication.
4. Clear the lysate by centrifugation and recover the supernatant.
5. Add Talon resin to the supernatant and purify in batch fashion following the manufacturer's protocol.

6. Check the purity of the isolated proteins by SDS-PAGE (95% purity based on Coomassie staining). Although not essential in our work, the molecular weights of each protein were verified by electrospray mass spectrometry.

3.3. Expression and Purification of Kinases

1. XD4 is a v-Src lacking the SH3 domain and the first 80 residues of the SH2 domain (a Δ[77-225] truncation of v-Src), and is highly active and suitable for prokaryotic expression.
2. Grow bacterial strain DH5α harboring pGEX-KT-XD4.
3. Harvest and lyse the cells as outlined above.
4. Purify XD4 from the lysate using glutathione–agarose beads ***(16,17)***.
5. Plasmid construction, expression, and purification of c-Abl are carried out similarly, as described ***(16,17)***.

3.4. PK Assays

3.4.1. XD4 (Src) Assay

1. Two sets of reactions are run in Src buffer with 2 µL purified XD4, varying amounts of GFP_{235}IYGEFG, and with or without 2.5 µCi [γ-^{32}P]ATP, at 30°C for 15 min. The total volume of each reaction was 20 µL.
2. At the end of the incubation, use one set of reactions (with [γ-^{32}P]ATP) for autoradiography, and use the second set of reactions (without [γ-^{32}P]ATP) for Western blotting.
3. For autoradiography, add 5 µL 53 Laemmli loading dye to each tube and heat the mixture at 90°C for 5 min.
4. Load sample solutions onto a 12% SDS-PAGE gel and perform electrophoresis until the bromophenol blue dye migrates out of the gel.
5. Stain the gel with Coomassie brilliant blue R-250.
6. Destain, dry, and expose the gel to X-ray film at room temperature.
7. For Western blotting, separate GFP_{235}IYGEFG from other proteins in the reaction mixture that may interfere with antiphosphotyrosine immunoblotting.
8. Mix 10 µL of Talon resin in 30 µL 20 m*M* Tris buffer, pH 8.0, and add the slurry to each reaction.
9. Separate the resin by brief centrifugation (15,000*g*), and wash 3X with the Tris buffer.
10. Elute GFP_{235}IYGEFG with 20 µL elution buffer and separate from the resin by brief centrifugation (15,000*g*).
11. Add 5 µL 53 Laemmli loading dye to each supernatant and heat the mixture at 90°C for 5 min. Spin the tubes to remove insoluble debris (15,000*g*, 5 min).
12. Load the sample solutions onto a 12% SDS-PAGE gels and perform electrophoresis until the bromophenol blue dye migrates out of the gel.
13. Transfer the proteins to a nitrocellulose membrane and incubate the membrane overnight in 5% dried milk in phosphate-buffered saline.
14. Probe the membrane with antiphosphotyrosine antibody 4G10, wash the blot, then visualize with horseradish peroxidase-conjugated secondary antibody using chemiluminescence ***(19)***.

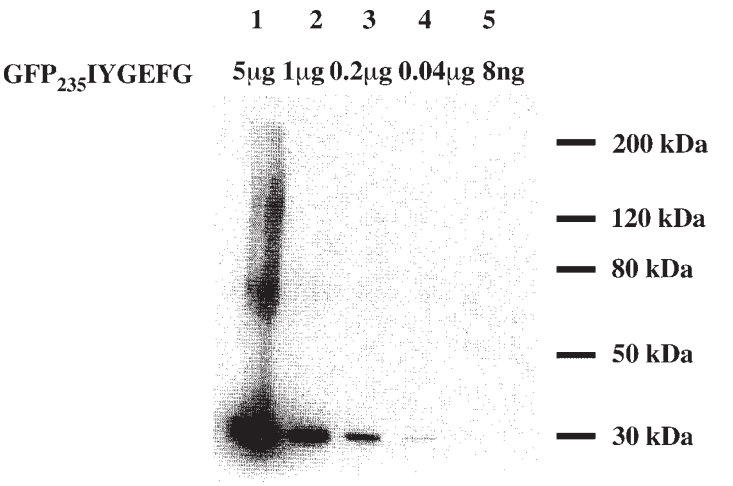

Fig. 2. Autoradiogram of XD4 phosphorylation reactions, using different amounts of GFP_{235}IYGEFG as indicated. Reactions were run in Src buffer with 2.5 μCi[γ-^{32}P]ATP for 15 min at 30°C. Laemmli loasing dye was added, and the mixture was heated to inactivate the kinase. Sample solutions were loaded onto a 12% SDS-PAGE gel, and electrophoresis was performed. The gel was dried and exposed to an X-ray film at room temperature. Exposure time was 14 h.

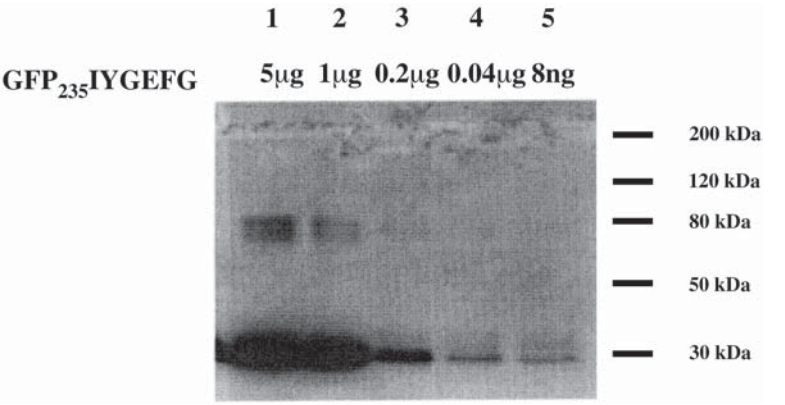

Fig. 3. Western blot of XD4 phosphorylation reactions with antiphosphotyrosine antibody, using different amounts of GFP_{235}IYGEFG. Reactions were run as described in **Fig. 2**, with the exception of [γ-^{32}P]ATP. GFP_{235}IYGEFG was separated from the reaction by addition of Talon resin (10 μL each reaction). The resin was separated and washed. GFP_{235}IYGEFG was eluted from the resin with 20 μL 200 m*M* imidazole in Tris buffer. Laemmli loading dye was added to the eluent. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and immunoblotted with antiphosphotyrosine antibody.

The abovementioned strategy was employed to construct the putative Src substrate, GFP_{235}IYGEFG. This construct was then tested for its ability to act as a Src substrate. As can be seen in the autoradiogram in **Fig. 1**, GFP alone is not a Src substrate (lane 1). Addition of the optimal Src phosphorylation sequence ***(10)*** IYGEFG to the C-terminus of GFP results in an exceedingly efficient Src substrate (lane 4), compared to the common Src substrate, activated enolase (lanes 2 and 3).

Figure 2 shows another autoradiogram using varying amounts of GFP_{235}IYGEFG in a Src kinase assay. This experiment demonstrates that phosphorylation of the GFP construct can be detected using nanogram quantities of GFP_{235}IYGEFG.

Figure 3 is a similar experiment used to determine whether this sensitivity can be duplicated using a generally less sensitive method, antiphosphotyrosine Western blotting. Again, the sensitivity is carried over and only 8 ng phosphoprotein can be detected, demonstrating that the phosphorylated tail of these GFP constructs is efficiently recognized by the monoclonal antibody, 4G10.

3.4.2. c-Abl Assay

1. Carry out duplicate reactions as described above, except c-Ab1 buffer and substrate (GFP_{235}AIYAAPF) are used, and the reaction is performed for 1 h at room temperature.
2. Resolve the samples by SDS-PAGE and transfer to a nitrocellulose membrane.
3. Visualize using antiphosphotyrosine antibody 4G10 and chemiluminescence.
4. GFP alone is not an Abl substrate (data not shown). However, appending the Abl optimal phosphorylation site, AIYAAPF at position 235 produced an excellent Abl substrate (**Fig. 4**).

3.4.3. PKA Assay

1. Reactions are run, and autoradiography is performed as in the XD4 assay. Exceptions are that a PKA buffer is used and the substrate is altered for PKA detection (GFP_{234}RRRRSII or GFP_{227}RRRRSII). Add 7 U PKA to each reaction in place of XD4.
2. Be sure to test unmodified GFP as a kinase substrate. This was done for PKA and GFP is shown to be a poor PKA substrate (**Fig. 5**, lane 6). However, simple C-terminal attachment of the PKA optimal phosphorylation sequence RRRRSII, from pGFP_{227}RRRSII, as identified by Zhou et al. ***(8)***, also proved to be a poor PKA substrate by this assay, so that pGFP_{227}RRRSII was produced and shown to be efficient (data not shown, *see* **Note 3**).

4. Notes

1. Previously, the last six C-terminal residues of GFP have been deleted without altering expression or fluorescence ***(20)***. The authors pursued the reasonable strat-

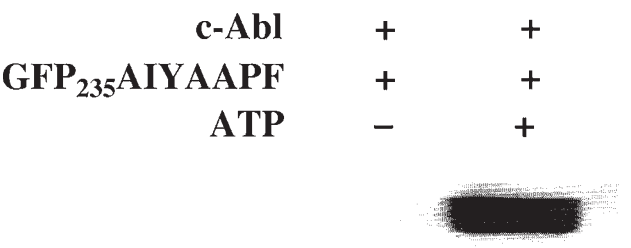

Fig. 4. Phosphorylation of GFP_{235}AIYAAPF by c-Abl. Each reaction was carried out with 50 m*M* Tris (pH 8.1), 10 m*M* $MgCl_2$, 200 μ*M* ATP, and 0.5 μg GFP_{235}AIYAAPF. The samples were resolved on an SDS-PAGE gel, and transferred to nitrocellulose membrane. Immunoblotting was performed with the antiphosphotyrosine antibody.

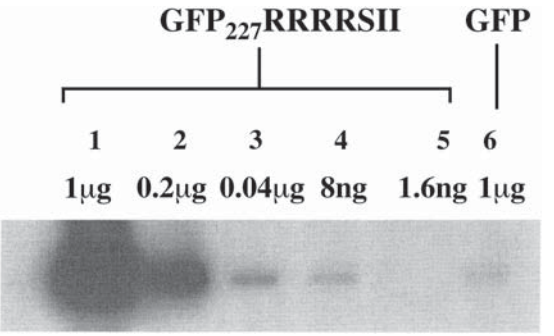

Fig. 5. Autoradiogram of PKA phosphorylation reactions with GFP_{235}RRRRSII. Each reaction was run in 50 m*M* Tris (pH 7.4), 10 m*M* $MgCl_2$, 20 μ*M* ATP, 2.5 μCi [γ-^{32}P]ATP, and different amounts of GFP_{235}RRRRSII or GFP, as labeled above each lane. PKA (7 U) was added, and the mixture was incubated for 15 min at 30°C. Laemmli loading dye was added and the reaction mixture was heated to inactivate the kinase. Electrophoresis was run on a 15% SDS-PAGE gel. The gel was dried and exposed to an X-ray film at room temperature for 14 h.

egy of attaching the phosphorylation sequence C-terminal to the last β strand, effectively replacing the abovementioned nonessential residues.

2. Creating an efficient GFP-based PK substrate for other kinases should follow a protocol analogous to those mentioned above. First, a peptide that has an optimal phosphorylation sequence must be identified. This can be determined experimentally ***(8,11)***, and the optimal phosphorylation sequences of many kinases are well known ***(8,9,11,14)***. Addition of this sequence to the C-terminus of GFP, using standard PCR techniques described in Materials and Methods, should rapidly generate an efficient kinase substrate. The success of this

procedure relies on the fact that GFP is a carrier protein for the peptide sequence, which remains unstructured.

3. Considering that the addition of this particular phosphorylation site also introduced a presumably efficient trypsin cleavage site into a known flexible region of the sequence, the protein product was subjected to mass spectrometry and was shown to be missing the phosphorylation sequence after the first arginine. To remedy this situation, the phosphorylation sequence was shifted to begin at position 227, closer to the last sequence containing secondary structure. GFP_{227}RRRRSII is resistant to proteolysis as judged by mass spectrometry and is an efficient PKA substrate (**Fig. 5**). Problems with proteolytic degradation as seen with the PKA substrate can be dealt with successfully by moving the peptide sequence a few positions toward the N-terminus, yielding a less flexible, proteolytically resistant protein.

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