Analytical Biochemistry **266,** 167–173 (1999)
Article ID abio.1998.2885, available online at http://www.idealibrary.com on IDEAL®

# Highly Efficient Green Fluorescent Protein-Based Kinase Substrates[1]

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Received October 24, 1997

**We have developed a general strategy for designing efficient protein substrates of protein kinases by attaching a phosphorylatable peptide sequence to the C-terminus of $His_6$-tagged green fluorescent protein (GFP). We found that several C-terminal attachment sites in GFP allow for correct presentation of the phosphorylatable tail to a variety of protein kinases. Using this strategy, we have constructed highly efficient GFP-based substrates for Src, c-Abl, protein kinase A, and protein kinase C βII protein kinases. The engineered GFP substrate for Src ($GFP_{235}$IYGEFG) is 300 times more efficient than the protein most commonly used as a Src substrate–rabbit muscle enolase.** 

Protein kinases are ubiquitous mediators of cellular signal transduction (1, 2). Their activity is tightly regulated in response to extracellular cues as well as intracellular signals. Many biochemical methods have been developed to study the level of kinase activation following receptor stimulation. Immunoprecipitation of protein complexes from detergent cell lysates followed by an assay for kinase activity is the most common of these methods. The immunoprecipitated kinase is usually resolved from other proteins in the complex via gel electrophoresis. One problem with this method is that if the immunoprecipitated complex does not contain an efficiently phosphorylatable substrate, the activity of the kinase can be difficult to detect. Often, exogenous substrates are added to immunoprecipitated kinase reactions to avoid this problem. The ideal substrate in such an *in vitro* kinase assay would be the natural cellular substrate of the kinase. Unfortunately, the specific cellular substrates of the majority of protein kinases have yet to be determined, making the development of such *in vitro* kinase assays problematic. This paper addresses the problem of generating highly efficient substrates for *in vitro* assays of protein kinases.

Traditionally, two types of substrates are used to measure protein kinase activity. Proteins fortuitously identified as substrates of the kinases of interest such as immunoglobulin heavy chain, rabbit muscle enolase, and histones are moderately good substrates (3, 4). Peptides derived from the screening of combinatorial peptide libraries are the most efficiently phosphorylated substrates (5–12), but they cannot be used in gel-based assays. Synthetic random polypeptides such as poly(Glu, Tyr) 4:1 have also been used in the development of efficient high-throughput assays for the c-Src protein kinase (13–15).

In the process of our efforts to engineer protein kinases with altered nucleotide specificity (16, 17), we recognized the need for versatile, highly efficient kinase substrates that could be used in nonradioactive kinase assays. We sought to convert the information gained from peptide libraries to design protein-based kinase substrates which are required for gel-based assay formats. The ideal kinase substrate would have the ability to serve as a specific substrate for particular kinases. It should be generalizable to diverse protein kinases, including both tyrosine kinases and serine/threonine kinases. It should be easy to prepare, versatile in terms of assay format, and inexpensive.

To achieve these goals, we extended the optimal peptide substrate sequence information obtained from combinatorial peptide libraries to the phosphorylation of a protein which can be separated cleanly from proteins in immune complex kinase reactions by poly-

[1] This work was supported by a National Science Foundation Early Career Development Award (MCB-9506929) and by the National Institutes of Health (1R011CA70331-01). K.M.S. is a Pew Scholar in the Biomedical Sciences.

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0003-2697/99 $30.00

**TABLE 1**

List of Phosphorylatable GFP Kinase Substrates

| Kinase[a] | GFP sequence (Beta sheet → Flexible) | Name |
|---|---|---|
| | $^{215}$RDHMVLLEFVTAAGITHGMDELYK$^{238}$ | GFP |
| Src | RDHMVLLEFVTAAGITHGMDEIYGEFGGS | $GFP_{235}$IYGEFG |
| Src | RDHMVLLEFVTAAEIYGEFG | $GFP_{227}$EIYGEFG |
| Ab1 | RDHMVLLEFVTAAGITHGMDAIYAAPF | $GFP_{235}$AIYAAPF |
| PKA | RDHMVLLEFVTAAGITHGMDRRRRSII | $GFP_{234}$RRRRSII |
| PKA | RDHMVLLEFVTAARRRRRSII | $GFP_{227}$RRRRSII |
| PKA | RDHMVLLEFVTAARRSII | $GFP_{227}$RRSII |

[a] Protein kinase which phosphorylates the optimal peptide substrate sequence (underlined) attached to GFP.

acrylamide gel electrophoresis (PAGE)[3] and detected via methods commonly used in kinase assays. We attached optimal substrate sequences to the C-terminus of a nonphosphorylatable host protein—green fluorescent protein (GFP). GFP can be easily produced in bacteria and purified via an attached $His_6$ tag at the N-terminus. We utilized a mutant GFP which possesses enhanced solubility, folding, and fluorescent properties over the wild-type protein (18). We asked whether such "tailed" GFP substrates could provide efficient substrates for assaying a variety of protein kinases such as Src, c-Abl, protein kinase A (PKA), and protein kinase C (PKC) $\beta$II in gel based nonradioactive assays.

## MATERIALS AND METHODS

Plasmid pGFPMut1, which contains the GFPMut1 gene in a pKEN vector, was a gift from B. P. Cormack (Stanford University). Plasmid vector pQE8 and the QIAEX II kit were purchased from Qiagen (Chatsworth, CA). Restriction enzymes, calf intestinal alkaline phosphatase (CIP), and T4 ligase were purchased from NEB (Beverly, MA). Oligonucleotide synthesis and automatic DNA sequencing were done in the Synthesis and Sequencing Facility at Princeton University. LB broth and LB agar were purchased from Bio101 (La Jolla, CA). PKA was purchased from Life Technology (Gaithersburg, MD). TALON resin was purchased from Clonetech (Palo Alto, CA). [$\gamma$-$^{32}$P]ATP was purchased from Du Pont NEN (Boston, MA). Antibody 4G10 was a gift from Brian Druker (Oregon Health Science Center, Portland, OR). SuperSignal chemiluminescent substrate for horseradish peroxidase was purchased from Pierce (Rockford, IL). Pfu polymerase was purchased from Stratagene (La Jolla, CA). Electrospray mass spectrometry was performed by the Mass Spectrometry Facility, Department of Chemistry, Princeton University, with a Hewlett–Packard 5989B spectrometer. Scintillation counting was carried out with a Beckman liquid scintillation spectrometer, Model LS5801.

[3] Abbreviations used: GFP, green fluorescent protein (in this paper, we refer to GFPMut1 as GFP); CIP, calf intestinal alkaline phosphatase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; PKC $\beta$II, protein kinase C $\beta$II isozyme; SDS, sodium dodecyl sulfate; XD4, v-Src lacking SH3 domain and the first 80 residues of the SH2 domain.

### *Procedures for Plasmid Construction*

Using the polymerase chain reaction (PCR), a *Bam*HI site was added to the 5′ end of GFPMut1 gene, and nucleotide sequence coding for the peptide of choice followed by a *Bam*HI site was added to the 3′ end of the gene. pGFPMut1 was used as the template. PCR was performed using Pfu polymerase under manufacturer's conditions. The PCR product was digested with *Bam*HI, separated by electrophoresis, and extracted using the QIAEX II kit. The vector pQE8, which contains a $His_6$ coding sequence before a *Bam*HI site, was digested with *Bam*HI, treated with CIP, separated, and extracted similarly. The vector and the insert were ligated using T4 ligase. The ligation mixture was used to transform competent JM109 *Escherichia coli*. Colonies were screened using a hand-held UV lamp, and green fluorescent colonies were picked. Plasmid DNA from these colonies were analyzed by restriction digest and positive clones were confirmed by sequencing.

*$pGFP_{235}IYGEFG$.* Forward primer, 5′-TCTAGGGATCCGGCATGAGTAAAGGA-3′; reverse primer, 5′-TCTAGGATCCGCCGAATTCGCCGTATATTTCATCCATGCCATG-3′. The resultant construct replaces the last three codons of GFP with **IYGEFGGS** (Table 1).

*$pGFP_{227}EIYGEFG$.* The forward primer is the same as that used for $pGFP_{235}$IYGEFG; reverse primer, 5′-TCTAGGATCCTTAGCCGAATTCGCCGTATATTTC-

AGCAGCTGTTACAAACTCAA-3′. The resultant construct replaces the last 11 codons of GFP with **IYGEFG** (Table 1).

*$pGFP_{235}AIYAAPF$.* The forward primer is the same as that used for $pGFP_{235}$IYGEFG; reverse primer, 5′-ACGTATTCGAATTAGAACGGCGCCGCATAGATCGCTTCATCCATGCCATGTGTAATC-3′. The resultant construct replaces the last three codons of GFP with **AIYAAPF** (Table 1).

*$pGFP_{234}RRRRSII$.* The forward primer is the same as that used for $pGFP_{235}$IYGEFG; reverse primer, 5′-GATAGGATCCTTAGATGATAGATCTAGGCCGGCGATCCATGCCATGTGTAATC-3′. The resultant construct replaces the last four codons of GFP with **RRRRSII** (Table 1).

*$pGFP_{227}RRRRSII$.* The forward primer is the same as that used for $pGFP_{235}$IYGEFG; reverse primer, 5′-TCTAGGATCCTTAGATGATAGATCTACGCCGGCGAGCAGCTGTTACAAACTCAA-3′. The resultant construct replaces the last 11 codons of GFP with **RRRRSII** (Table 1).

*$pGFP_{227}RRSII$.* 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.

### *Expression and Purification of GFP*

*E. coli.* strain JM109 harboring one of the constructs was grown overnight at 37°C in LB broth with 75 $\mu$g/ml ampicillin. The culture was diluted 1:100 into 50 ml of same medium and grown at 37°C until $OD_{596}$ reached 0.4 (about 3 h). Isopropyl $\beta$-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and the culture was grown for 8–10 h. The cells were harvested by centrifugation and lysed by sonication. The lysate was cleared by centrifugation and the supernatant was recovered. TALON resin was added to the supernatant and purification was done in batch fashion following the manufacturer's protocol. The purity of the proteins was checked by sodium dodecyl sulfate (SDS)–PAGE (>95% purity based on Coomassie staining). The molecular weights of each protein were verified by electrospray mass spectrometry.

### *Expression and Purification of XD4*

Bacterial strain DH5$\alpha$ harboring pGEX-KT-XD4 was grown, harvested, and lysed as outlined above. XD4 was purified from the lysate using glutathione–agarose beads as described (16).

Plasmid construction, expression, and purification of c-Abl were carried out as described (16). c-Abl is a fusion protein of GST and Abl catalytic domain in which threonine 315 was mutated to a glycine.

### *Activation of Rabbit Muscle Enolase (3)*

Equal volumes of the enolase (5 mg/ml) and acetic acid (50 mM) were mixed. The mixture was incubated at 30°C for 5 min.

### *XD4 Assay*

Two sets of reactions were run in Src buffer (100 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 $\mu$g/ml bovine serum albumin, 0.1 mM dithiothreitol 1 mM sodium orthovanadate, 0.2 mM ATP) with 2 $\mu$l XD4 and various amounts of $GFP_{235}$IYGEFG, with or without 2.5 $\mu$Ci [$\gamma$-$^{32}$P]ATP, at 30°C for 15 min. The total volume of each reaction was 20 $\mu$l. At the end of the incubation, one set of reactions (with [$\gamma$-$^{32}$P]ATP) was used for autoradiography and the second set of reactions (without [$\gamma$-$^{32}$P]ATP) was used for Western blotting. For autoradiography, 5 $\mu$l of 5× Laemmli loading dye was added to each tube and the mixture was heated at 90°C for 5 min. Sample solutions were loaded onto a 12% SDS–PAGE gel and electrophoresis was performed until bromophenol blue dye migrated out of the gel. The gel was stained with Coomassie brilliant blue R-250, destained, dried, and exposed to an X-ray film at room temperature. For Western blotting, $GFP_{235}$IYGEFG was separated from other proteins in the reaction mixture which interfered with anti-phosphotyrosine immunoblotting. Ten microliters of TALON resin in 30 $\mu$l Tris buffer (20 mM Tris, pH 8.0) was added to each reaction and mixed. The resin was separated by brief centrifugation (15,000*g*) and washed three times with the Tris buffer. $GFP_{235}$IYGEFG was eluted with 20 $\mu$l of elution buffer (the Tris buffer plus 200 mM imidazole) and separated from the resin by brief centrifugation (15,000*g*). Five microliters of 5× Laemmli loading dye was added to each supernatant and the mixture was heated at 90°C for 5 min. The tubes were centrifuged (15,000*g*, 5 min). The sample solutions were loaded onto a 12% SDS–PAGE gels and electrophoresis was run until the bromophenol blue dye migrated out of the gel. The proteins on the gel were transferred to a nitrocellulose membrane and the membrane was incubated overnight with 5% dried milk in phosphate-buffered saline. The membrane was probed with anti-phosphotyrosine antibody 4G10 and then visualized with horseradish peroxidase-conjugated secondary antibody using chemiluminescence.

### *PKA Assay*

Reactions were run and autoradiography was performed similarly to the XD4 assay except for the use of a different buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$), ATP concentration (20 $\mu$M), substrate ($GFP_{234}$RRRRSII or $GFP_{227}$RRRRSII), and the kinase (PKA, 7 units in each reaction).

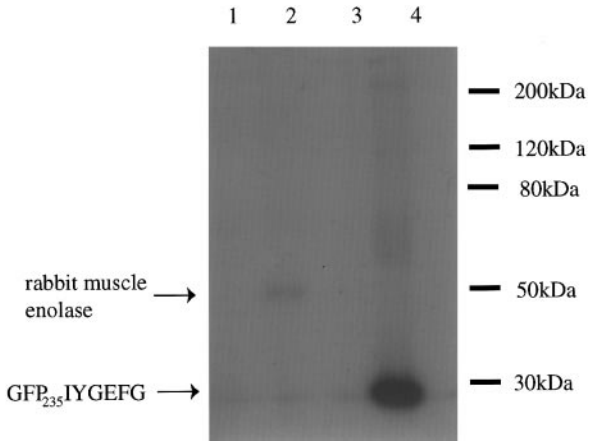


**FIG. 1.** Comparison of GFP, enolase, and $GFP_{235}$IYGEFG as XD4 substrates in a radioactive assay. Lane 1, 1 $\mu$g GFP; lane 2, 5 $\mu$g activated enolase; lane 3, 5 $\mu$g enolase; lane 4, 0.5 $\mu$g $GFP_{235}$IYGEFG. Reactions were run as described under Materials and Methods. Samples were run 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.

### *c-Abl Assay*

Each reaction was carried out with 50 mM Tris (pH 8.1), 10 mM $MgCl_2$, 200 $\mu$M ATP, and 0.5 $\mu$g $GFP_{235}$AIYAAPF for 1 h at room temperature. The samples were resolved on an SDS–PAGE gel and transferred to a nitrocellulose membrane. Visualization was carried out using anti-phosphotyrosine antibody 4G10 and chemiluminescence (19).

## RESULTS

### *Test of GFP as a Host Protein*

The ideal host protein to attach phosphorylatable sequence motifs to would contain no phosphorylation sites of its own and yet would contain a site where a phosphorylatable 6- to 10-amino acid sequence could be attached in an exposed part of the protein. Indeed, GFP is not a substrate for XD4, a v-Src catalytic domain fusion protein, as shown in Fig. 1 (lane 1). We analyzed the structure of GFP to determine an appropriate site to attach a peptide sequence for v-Src phosphorylation (20). Dopf and Horiagon showed that up to six residues from the C-terminus could be deleted without disrupting GFP fluorescence (21). Since the C-terminal tail of GFP is thought to be unstructured, we decided to attach the phosphorylatable tails to the C-terminus rather than the N-terminus of GFP. Zhou *et al.* (7) found that the peptide sequence EIYGEFG was the optimal peptide substrate for Src by screening a combinatorial peptide library for efficient Src substrates. The C-terminus of GFP contains the sequence ELYK (Table 1). We guessed that by eliminating the last three amino acids of GFP we would not disrupt the folding or fluorescent properties of GFP. The new C-terminal residue is then glutamate, which is the first residue in the optimal peptide sequence we wanted to attach to GFP, thus minimizing the number of amino acid changes necessary to produce the desired GFP construct, termed $GFP_{235}$IYGEFG (Table 1).

### *Efficiency of $GFP_{235}$IYGEFG Phosphorylation by Src*

We first tested $GFP_{235}$IYGEFG as a substrate of XD4. The $GFP_{235}$IYGEFG protein is an excellent substrate for XD4, as shown in Fig. 1 (lane 4). We compared the efficiency of $GFP_{235}$IYGEFG phosphorylation with that of rabbit muscle enolase, a commonly used Src kinase substrate, by XD4. Figure 1 shows enolase is a very poor substrate without activation (lane 3) and only a modest substrate after activation (lane 2). In contrast, $GFP_{235}$IYGEFG is an excellent substrate, being 300 times better than the activated enolase (comparison based on phosphorylation per protein molecule as measured by scintillation counting).

Figure 2 shows the autoradiogram of reactions using different amounts of $GFP_{235}$IYGEFG and [$\gamma$-$^{32}$P]ATP. Phosphorylation of $GFP_{235}$IYGEFG can be detected with as little as 8 ng of this protein after overnight exposure of the gel at room temperature without using an intensifying screen. Since $GFP_{235}$IYGEFG is a particularly good substrate for Src, we asked whether a nonradioactive assay method, which is considerably less sensitive than those using [$\gamma$-$^{32}$P]ATP, would detect Src's activity. Figure 3 shows the result of an anti-phosphotyrosine Western blot of kinase reactions containing the same amounts of $GFP_{235}$IYGEFG used in the radioactive assay (Fig. 2). The sensitivity of the

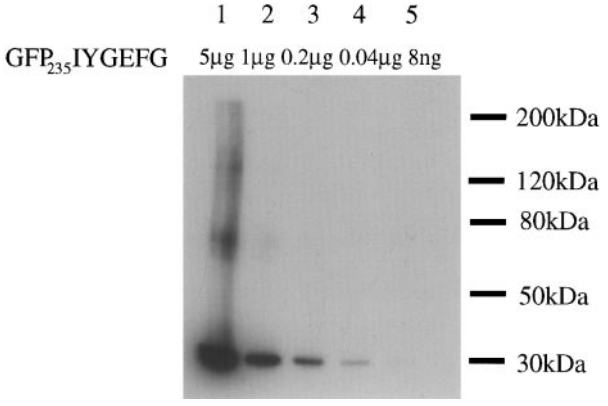


**FIG. 2.** Autoradiogram of XD4 phosphorylation reactions using different amounts of $GFP_{235}$IYGEFG as indicated. Reactions were run in Src buffer with 2.5 $\mu$Ci [$\gamma$-$^{32}$P]ATP for 15 min at 30°C. Laemmli loading dye was added and the mixture was heated to inactivate the kinase. Sample 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.

nonradioactive assay is comparable to the radioactive assay, in that as little as 8 ng of the protein can be detected by immunoblotting with the anti-phosphotyrosine antibody. The higher molecular weight bands in the immunoblot are aggregates of $GFP_{235}$IYGEFG (22).

*Phosphorylatable GFP for Abl*

To determine if GFP kinase substrates could be engineered for other tyrosine kinases, we prepared a GFP substrate using the known optimal peptide substrate for c-Abl, a non-Src family protein tyrosine kinase. We first asked whether GFP itself was a substrate for c-Abl. As with Src, GFP was not phosphorylated by c-Abl (data not shown). To construct a phosphorylatable GFP substrate for c-Abl, we replaced the last three codons of GFP with AIYAAPF (Table 1). Since no alanines are present in the last eight amino acids of GFP, we could not utilize a portion of GFP to construct a c-Abl substrate as we did with Src substrate $GFP_{235}$IYGEFG. As shown in Fig. 4, $GFP_{235}$AIYAAPF is an excellent substrate for Abl.

*Phosphorylatable GFP for PKA*

We next asked if this approach could be extended to serine/threonine kinases. We tested GFP as a substrate for PKA, the prototypical serine/threonine kinase, to ensure that no site in GFP itself was phosphorylated by this kinase. Indeed, only very minimal phosphorylation of GFP by PKA was observed (Fig. 5, lane 6). Zhou *et al.* identified RRRRSII as the optimal peptide substrate for PKA (8). We constructed a GFP substrate for PKA, $GFP_{234}$RRRRSII, in which the last four residues of GFP were replaced by RRRRSII. However, this GFP substrate for PKA was poorly phosphorylated by PKA (data not shown). Since the optimal substrate sequence for PKA includes many arginine residues which are efficient cleavage sites for trypsin-like proteases, we wondered whether $GFP_{234}$RRRRSII was being degraded by bacterial proteases. We used electrospray mass spectrometry to determine if bacterial proteases cleaved the phosphorylatable tail from $GFP_{234}$RRRRSII. The predicted molecular weight for full-length $GFP_{234}$RRRRSII is 28554.4, whereas the molecular weight obtained by electrospray mass spectrometry is 27785.2, suggesting all but the first arginine of the amino acids added were degraded.[4]

We then analyzed the C-terminus of GFP for another site to append a phosphorylatable sequence which might be protected from degradation. We reasoned that since wild-type GFP was stable to bacterial proteases, perhaps attachment of the Arg-rich optimal PKA substrate sequence closer to a secondary structural element of GFP might lead to a more stable protein. That wild-type GFP is stable to trypsin and chymotrypsin further suggested this strategy might be successful (23). We constructed $GFP_{227}$RRRRSII (Table 1) and asked if the purified protein was degraded. Electrospray analysis gave a molecular weight of 28009.2 (predicted molecular weight 28014.7), indicating that this protein was not being degraded by cellular proteases despite having four consecutive arginine residues. As shown in Fig. 5, $GFP_{227}$RRRRSII is an excellent substrate for PKA. Furthermore, low concentrations of $GFP_{227}$RRRRSII can be detected in the autoradiogram. Unfortunately, high-affinity phosphoserine specific antibodies are not available, making nonradioactive immunoblot assays for serine/threonine kinases problematic.

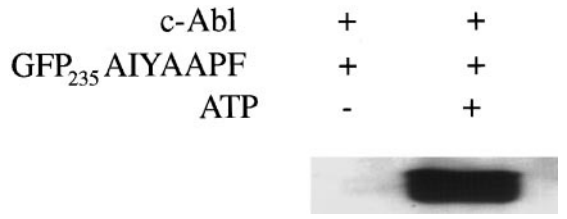


**FIG. 4.** Phosphorylation of $GFP_{235}$AIYAAPF by c-Abl (containing a T315G mutation which does not affect catalysis). Each reaction was carried out with 50 mM Tris (pH 8.1), 10 mM $MgCl_2$, 200 $\mu$M ATP, and 0.5 $\mu$g $GFP_{235}$AIYAAPF. The samples were resolved on an SDS–PAGE gel and transferred to nitrocellulose membrane. Immunoblotting was performed with the anti-phosphotyrosine antibody.

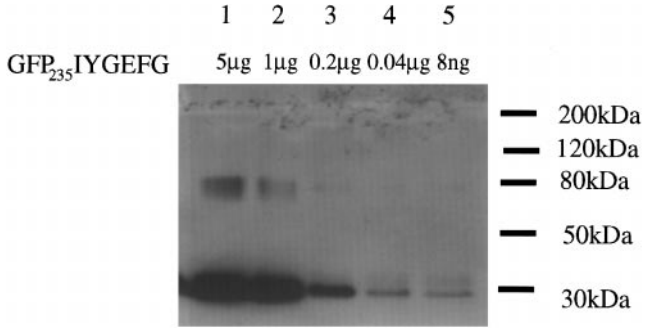


**FIG. 3.** Western blot of XD4 phosphorylation reactions with anti-phosphotyrosine antibody using different amounts of $GFP_{235}$IYGEFG. Reactions were run as described in the legend to Fig. 2, with the exception of [$\gamma$-$^{32}$P]ATP. $GFP_{235}$IYGEFG was separated from the reaction by addition of TALON resin (10 $\mu$l each reaction). The resin was separated and washed. $GFP_{235}$IYGEFG was eluted from the resin with 20 $\mu$l of 200 mM 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 anti-phosphotyrosine antibody.

[4] We also carried out purification of $GFP_{234}$RRRRSII in the presence of protease inhibitors. Molecular weight analysis by electrospray MS indicated that last two isoleucine residues were absent.

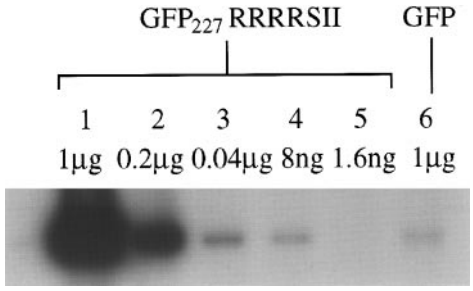


**FIG. 5.** Autoradiogram of PKA phosphorylation reactions with $GFP_{227}$RRRRSII. Each reaction was run in 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 20 mM ATP, 2.5 $\mu$Ci [$\gamma$-$^{32}$P]ATP, and different amounts of $GFP_{235}$RRRRSII or GFP as labeled above each lane. PKA (7 units) was added and the 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 a X-ray film at room temperature for 14 h.

### *Distance between the Added Sequence and the GFP Core Structure Determines Phosphorylation Efficiency*

Since PKA was able to phosphorylate an optimal sequence close to the core of GFP, we wondered whether the protein tyrosine kinase Src could also carry out the phosphorylation reaction when the phosphorylatable sequence is close to the core of GFP. We constructed $GFP_{227}$EIYGEFG (Table 1), which moved the phosphorylatable sequence seven residues closer to the core of GFP than that in $GFP_{235}$IYGEFG. Surprisingly, this construct is not a Src substrate at all (data not shown).

## DISCUSSION

In searching for a versatile, nonradioactive kinase assay system, we sought to combine the advantages of highly efficient peptide substrate sequences with the convenience of protein substrates useful in PAGE-based kinase assays. We chose GFP 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 compact protein which has a few flexible regions except its C-terminal tail. Since traditional eukaryotic protein kinases are thought to phosphorylate unstructured regions of proteins (24), we predicted wild-type GFP itself would not be a substrate for any protein kinases. Indeed, GFP is not a substrate for any kinase we tested. Finally, GFP's intrinsic fluorescence is a very useful property in identification of bacterial transformants which express the desired GFP–peptide fusion protein. This makes the production of dozens of such GFP based substrates for the more than 2000 hypothesized kinases much easier because of the simple detection of GFP kinase substrate-expressing colonies. After construction, the availability of phosphorylatable protein is virtually unlimited and the cost is minimal, unlike most commercially available protein kinase substrates, including synthetic peptides. Other small, stably folded proteins can also serve as host proteins, provided that they are not phosphorylated by protein kinases.

Our data demonstrate that the phosphorylatable sequences added to the C-terminus of GFP are flexible enough to allow for efficient phosphorylation by protein kinases. This was true for all four classes of kinases tested (PKC $\beta$II data not shown). The superiority of phosphorylatable GFP as a kinase substrate over commonly used generic protein substrates is shown by its 300 times higher level of phosphorylation by Src compared to rabbit muscle enolase. Furthermore, in some cases, substrate sequences added to the C-terminus of GFP are specific for different protein kinases. For example, $GFP_{235}$IYGEFG is an excellent substrate for Src but a poor substrate for c-Abl (data not shown). This agrees with results from assays using peptide substrates with identical sequences and the same kinases (7). We also demonstrate that phosphorylatable GFP can be used in nonradioactive assays such as anti-phosphotyrosine Western blotting with sensitivity comparable to radioactive assays. Applicability of this method to nonradioactive kinase assays is particularly useful for studying mutant kinases which use nonnatural nucleotide substrates which are not commercially available in $^{32}$P-labeled form. Since our GFP mutants contain a single phosphorylation site, they could serve as substrates for kinetic studies of protein kinases when use of a protein substrate is desirable (25). We did find, however, that at high concentration (>5 $\mu$g per lane), GFP became aggregated (Figs. 2 and 3), which may complicate such kinetic experiments.

The GFP-based kinase substrates might be useful in high-throughput kinase assays. One possible format is to phosphorylate the GFP substrate with [$\gamma$-$^{32}$P]ATP and then use trichloroacetic acid to precipitate the GFP substrate and quantitate the radioactivity contained in the pellet. This type of precipitation assay cannot be carried out using conventional peptide substrates. Another approach could be to immobilize the $His_6$-GFP substrate to a plate using anti-GFP antibodies or nitrilotriacetic acid $Ni^{2+}$-derivatized plates and to quantitate the level of phosphorylation using an anti-phosphotyrosine antibody. A third approach that takes advantage of the fluorescent properties of GFP itself would be to use anti-phosphotyrosine-coated plates to capture the phosphorylated GFP. The phosphorylated GFP could be quantitated using a fluorescence plate reader.

An appealing possibility for using a fluorescent protein as a kinase substrate would be to have phosphor-

ylation status affect protein fluorescence in some way. This might enable one to detect phosphorylation events in whole cells in a similar fashion to the recently developed GFP-based sensor of intracellular $Ca^{2+}$ levels (26). None of the GFP kinase substrates we have made displays altered fluorescent properties (absorbance/emission maxima or intensity) upon phosphorylation (data not shown). This is not surprising considering that we attached the phosphorylatable tail to the flexible portion of the host protein. Most GFP mutants which display altered fluorescent properties do so by altering hydrogen bonding to the chromophore buried inside the "barrel" of the protein or by mutations to the chromophore itself (20, 23). Since this region of the protein is completely sequestered from solvent it is difficult to imagine a strategy to phosphorylate residues in this region of the protein to cause a fluorescence change.

The addition of phosphorylatable sequences to structurally defined sites in proteins such as GFP might be used to elucidate the complex relationship between the secondary structure and the primary sequence which controls protein phosphorylation (24). Phosphorylatable GFP variants which have the same phosphorylatable sequence at different distances from secondary structural elements of GFP may serve as probes for finding answers to these questions. For example, we asked if protein tyrosine and serine/threonine kinases had different requirements for how close the phosphorylatable residue could be to a $\beta$-sheet. We found that $GFP_{227}$EIYGEFG is not a substrate for Src (data not shown), and yet $GFP_{227}$RRSII is an excellent substrate for PKA. Both substrates have the phosphorylatable residue at position 230 of GFP, and yet the tyrosine kinase cannot phosphorylate the substrate while the serine/threonine kinase can. This observation suggests that accessibility of catalytic sites in protein tyrosine and serine/threonine kinases may be quite different. The hydroxyl group on tyrosine is far more distant from the peptide backbone compared to that of serine/threonine. The failure of $GFP_{227}$EIYGEFG to act as a Src substrate may indicate that the catalytic site of Src is more sensitive to steric factors than that of PKA. Importantly, both $GFP_{227}$EIYGEFG and $GFP_{227}$RRSII proteins have identical fluorescence spectra. This serves as a convenient check on the integrity of the secondary structure of the protein and is another advantage of using GFP as the host protein.

In summary, we have found a kinase substrate system that utilizes information from peptide libraries to create efficiently phosphorylatable proteins which are easy to express and purify. These novel protein kinase substrates allowed us to identify protein kinase mutants with altered nucleotide specificity using nonradiolabeled nucleotide analogs (16, 17).

## ACKNOWLEDGMENTS

We thank B. P. Cormack, B. Druker, A. Toker (Harvard), and J. Y. Wang (UCSD) for GFP mutant constructs, antibody 4G10, PKC $\beta$II plasmid, and c-Abl plasmid, respectively. We thank K. Shah, S. Leibler, M. G. Surette, T. Surrey, and M. B. Elowitz for helpful discussions and comments and L. Witucki for help in designing optimal peptide sequences.

## REFERENCES

1. Hunter, T. (1995) *Cell* **80,** 225–236.
2. Cohen, G. B., Ren, R., and Baltimore, D. (1995) *Cell* **80,** 237–248.
3. Cooper, J. A., Esch, F. S., Taylor, S. S., and Hunter, T. (1984) *J. Biol. Chem.* **259,** 7835–7841.
4. Casnellie, J. E., and Krebs, E. G. (1984) *Adv. Enzyme. Regul.* **22,** 501–515.
5. Wu, J., Ma, Q. N., and Lam, K. S. (1994) *Biochemistry* **33,** 14825–14833.
6. Till, J. H., Annan, R. S., Carr, S. A., and Miller, W. T. (1994) *J. Biol. Chem.* **269,** 7423–7428.
7. Zhou, S., Carraway, K. L., III, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995) *Nature* **373,** 536–539.
8. Zhou, S., Blechner, S., Hoagland, N., Hoekstra, M. F., Piwnica-Worms, H., and Cantley, L. C. (1994) *Curr. Biol.* **4,** 973–982.
9. Dente, L., Vetriani, C., Pelicci, G., Lanfrancone, L., Pelicci, P. G., and Cesareni, G. (1997) *J. Mol. Biol.* **269,** 694–703.
10. Nishi, T., Budde, R. J., McMurray, J. S., Obeyesekere, N. U., Safdar, N., Levin, V. A., and Saya, H. (1996) *FEBS Lett.* **399,** 237–240.
11. Schmitz, R., Baumann, G., and Gram, H. (1996) *J. Mol. Biol.* **260,** 664–677.
12. Chan, P. M., Keller, P. R., Connors, R. W., and Leopold, W. R., and Miller, W. T. (1996) *FEBS Lett.* **394,** 121–125.
13. Braun, S., Raymond, W. E., and Racker, E. (1984) *J. Biol. Chem.* **259,** 2051–2054.
14. Braunwalder, A. F., Yarwood, D. R., Sills, M. A., and Lipson, K. E. (1996) *Anal. Biochem.* **238,** 159–164.
15. Braunwalder, A. F., Yarwood, D. R., Hall, T., Missbach, M., Lipson, K. E., and Sills, M. A. (1996) *Anal. Biochem.* **234,** 23–26.
16. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3565–3570.
17. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K. M. (1998) *Chem. Biol.* **5,** 91–101.
18. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) *Gene* **173,** 33–38.
19. Friedman, J. D. Ph.D. Thesis, Department of Molecular Biology, Princeton University, 1997.
20. Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Reminston, S. J. (1996) *Science* **273,** 1392–1395.
21. Dopf, J., and Horiagon, T. M. (1996) *Gene* **173,** 39–44.
22. Morise, H., Shimomura, S., Johnson, F. H., and Winaut, J. (1974) *Biochemistry* **13,** 2656–2662.
23. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) *Trends Biochem. Sci.* **20,** 448–455.
24. Johnson, L. N. (1993) *Annu. Rev. Biophys. Biomol. Struct.* **22,** 199–232.
25. Sondhi, D., Xu, W., Songyang, Z., Eck, M. J., and Cole, P. A. (1998) *Biochemistry* **37,** 165–172.
26. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) *Nature* **388,** 882–887.